# Maillard-Induced Glycation of Whey Protein Hydrolysate to Increase Solubility and Thermal Stability and Reduce Allergenicity in Acidified Protein Beverages

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## Dedication

This thesis is dedicated to my Mom and Dad who always supported me no matter what I wanted to do. I couldn't have asked for better parents.

## Abstract

Interest in whey protein is continually increasing globally as a result of its excellent nutritive value, unique physiological benefits, and diverse functionality. More specifically, whey protein hydrolysates (WPH) are value-added ingredients that are experiencing a rapid increase in usage and market volume in part due to their enhanced functional properties and physiological benefits. However, maintaining quality and shelf-life stability as well as increasing concern over whey protein allergenicity are major hurdles that hinder the use of biologically active WPH in beverages. Maillard-induced glycation has been shown to be a novel protein modification technique with potential to address these challenges. It is hypothesized that a low degree of hydrolysis in addition to limited and controlled Maillard-induced glycation will enhance solubility and thermal stability while maintaining nutritional and physiological quality, and synergistically reducing allergenicity of whey protein.

Thus, the objectives of this study were twofold: (1) to produce and assess solubility and thermal stability of a partially-glycated whey protein hydrolysate product using controlled and limited Maillard conditions, and (2) to assess the effects on nutritional quality, bioactivity (anti-hypertensive activity) and allergenicity.

Whey protein hydrolysate was reacted with dextran over 12-120 h of incubation at 60°C, 0.49 water activity ($a_w$), and a 4:1 ratio of dextran to protein to produce partially-glycated WPH (PGWPH). Extent of glycation was monitored via estimation of Amadori compound formation, fluorescent compound formation, browning, free amino group loss, and visualization of protein/peptide molecular weight distribution following sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Glycation was initiated as early as 12 h of incubation. Partial glycation and minimal browning were maintained through 120 h of incubation, and loss of free amino groups was between 5-30%. Based on the results coupled with feasibility purposes, a 48 h incubation time period was selected for further analysis, as this time point resulted in modest Amadori compound formation and % amino group blockage (21.8%), with minimal progression to

intermediate and advanced stages of the Maillard reaction. Free, unreacted dextran was then removed from the 48 h incubated sample using ultrafiltration and hydrophobic interaction chromatography (HIC) to produce purified PGWPH. The final composition of purified PGWPH was approximately 88% protein and 12% carbohydrate.

Solubility and thermal stability of PGWPH were assessed at 5% protein concentration prior and post heat treatment at 80°C for 30 min. Lysine blockage was assessed using a furosine assay, and digestibility was determined using a sequential pepsin-trypsin *in-vitro* digestibility assay. The antihypertensive activity was determined by measuring the angiotensin converting enzyme (ACE) inhibitory activity. Allergenicity was determined following an indirect ELISA using sera from milk sensitive donors.

Partial glycation of WPH resulted in enhanced solubility and thermal stability, particularly near the isoelectric point (pI), where PGWPH remained soluble after heating while WPH lost over 50% of its solubility. Changes in surface hydrophobicity and free sulfhydryl groups were minimal upon heating. The enhanced solubility and thermal stability of PGWPH, even when the pH was close to the pI of the whey protein, was attributed to the resistance to denaturation and structural modifications. Nutritional quality and bioactivity of WPH was minimally impacted upon partial glycation, as lysine blockage was only ~2%, and digestibility (58.7%) and antihypertensive activity of PGWPH ($IC_{50}$ =0.249) were similar to that of WPH. However, allergenicity of WPH was not further reduced upon partial glycation.

Overall, this work has shown for the first time that partial Maillard-induced glycation can be induced and controlled to low-levels in WPH, producing a value-added product with enhanced solubility and thermal stability, as well as maintained nutritional quality and bioactivity. Acidified whey protein beverages formulated with PGWPH in place of WPH or WPI may have a longer shelf life, a more acceptable flavor, and protein content greater than 4.2%, allowing for a "high protein" beverage claim to be made. In turn, the utilization of biologically active WPH in acidified protein beverages would

greatly increase, and consumer demands for a functional, high protein beverage could be fulfilled.

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# 1. Literature Review

## 1.1. Introduction and Objectives

Interest in whey protein is continually increasing globally as a result of its excellent nutritive value, unique physiological benefits, and diverse functionality. Whey protein is an excellent source of essential amino acids, as well as a rich source of branched chain amino acids and sulfur containing amino acids. Its physiological benefits include gastrointestinal functions, anticarcinogenicity, antimicrobial activity, growth promotional activity, and immunoactivity (Gutteridge et al., 1981; Van Beresteijn et al., 1994; Caccavo et al., 2002; Ha and Zemel, 2003). Furthermore, the physiochemical characteristics of whey protein render it versatile in functionality, such as water binding, emulsification, foaming and gelation.

Worldwide, whey protein is becoming increasingly abundant as a result of the growth in cheese production along with the advancements in membrane separation and concentration technologies. The abundance of whey protein along with the technological developments in the utilization of whey protein resulted in the broadening of the types and number of food based applications of whey and derived whey protein products. Such applications include dairy products, baked goods, snack foods, processed meats, candies, infant formulas, protein bars and beverages.

Enzymatic production and use of functional and bioactive whey protein hydrolysates (WPH) have gained prominence in the food industry due to increased consumer awareness and demand of healthy foods. Therefore, the food industry is facing the challenge of choosing healthier ingredients that are economical and can sustain or even improve texture and overall quality of the final product. Whey protein hydrolysates are an excellent solution to this problem as they have enhanced functional and biological properties compared to their native counterpart.

Whey protein acidic beverages, in particular, are gaining popularity in the market, targeting athletes and health conscientious individuals. While whey proteins are reasonably soluble in acidic beverages (pH~3.4), upon thermal processing and extended

storage, protein aggregation and deterioration of overall quality will occur. As a result, whey protein acidified beverages currently available on the market have a short shelf life and a protein content less than 4%, which is below the minimum percentage (4.2%) required by the FDA to claim a “high protein beverage” (21 CFR 101.54 B). Although solubility is improved upon whey protein hydrolysis, sensory quality and thermal stability remain issues that hinder its use in beverages and other food applications.

Another obstacle preventing the expanded use of whey protein ingredients is their allergenicity. Milk protein, including whey, ranks among the “Big 8” food allergens (FDA, 2014). Together with peanut, soy, egg, fish, shellfish, treenuts and wheat, these "Big 8" allergenic foods account for more than 90% of allergic reactions in consumers (Boyce et al., 2010). Currently in the United States, it is estimated that 5.1% of young children and 3-4% of adults are affected by food allergies (Branum and Lukacs, 2008; Jackson et al., 2013). Food allergy is highest for peanut (2.5%), followed by milk (1.3%) (Liu et al., 2010; Gupta et al., 2011). Furthermore, the prevalence of food allergies appears to be on the rise. In 2008, the Center for Disease Control and Prevention (CDC) reported that the prevalence of food allergies among children increased 18% during 1997–2007 (Branum and Lukacs, 2008). In 2013, the CDC reported that food allergies increased 50% between 1997 and 2011 (Jackson et al., 2013). Based on this pattern, it is projected that whey protein allergy prevalence will continue to increase in the future.

Many techniques have been used to alter or modify the structure of the whey protein in order to develop hypoallergenic whey protein ingredients. Such techniques include heat treatment, enzymatic hydrolysis, fermentation and sugar conjugation via the Maillard reaction. Excessive hydrolysis of whey protein leads to reduction in allergenicity (Lee, 1992; Van Beresteijn et al., 1994). However, excessively hydrolyzed whey protein has limited food applications due to poor sensory quality from the release of bitter peptides and loss of functionality and shelf-life stability. Similarly, Maillard-induced glycation of whey protein has been shown to significantly reduce allergenicity (Kobayashi et al., 2001; Bu et al., 2010; Li et al., 2011). However, extensive glycation

may lead to nutritional loss due to lysine blockage, and loss of functionality. Moreover, the conditions employed may be unfeasible for full scale production in the food industry.

It is hypothesized that a low degree of hydrolysis in addition to limited and controlled Maillard-induced glycation will have a synergistic effect on the reduction of allergenicity of whey protein while maintaining nutritional value and enhancing functionality. Limited hydrolysis of whey protein will disrupt some conformational and linear epitopes to decrease allergenicity, as well as result in the release of bioactive peptide, which exert a number of physiological benefits. Limited Maillard-induced glycation under controlled conditions will further reduce allergenicity, enhance protein functionality, namely solubility, and limit nutritive loss and the formation of advanced glycation end products.

Therefore, the objective of this research was to develop a hypoallergenic and bioactive whey protein ingredient with improved solubility using a combination of limited enzymatic hydrolysis and controlled Maillard-induced glycation. This work will provide information about the combined effect of limited enzymatic hydrolysis and controlled Maillard-induced glycation on the allergenicity, bioactivity and solubility of whey protein. The specific objectives for this work are:

1) Determine the solubility and thermal stability of bioactive WPH subjected to limited and controlled Maillard-induced glycation.
2) Determine the nutritional quality, bioactivity (anti-hypertensive activity) and allergenicity of partially glycated WPH.

### 1.2. Market Significance of Whey Protein

Whey is the liquid byproduct of cheese and casein production containing nutritive and diversely functional proteins, as well as lactose, vitamins, minerals, and fat. However, managing the vast volume of whey produced by the cheese industry can be a problem. For every 1 kg of cheese, 9 kg of liquid whey is generated, totaling over 108

million metric tons of cheese whey worldwide each year, with over half produced in the United States alone (Bylund, 1995; Prazeres et al., 2012; Mollea et al., 2013). Such a large surplus of whey poses a disposal challenge for economical and environmental reasons. In the past, most cheese whey was disposed of by land application or deposited into water streams leading to lakes, rivers and oceans. However, whey can be harmful to the natural environment as its decomposition causes oxygen consumption, eutrophication and toxicity in the receiving environment (Prazeres et al., 2012). To reduce this waste problem and take advantage of valuable nutrients in whey, new food applications for added value products such as lactose powder, whey powder, and whey proteins have been developed, leading to advancements in processing and separation technologies to recover and concentrate the valuable components of whey. Different methods of recovery and treatment include membrane and chromatographic separation processes in addition to both precipitation and complexing techniques (Bylund, 1995). According to nutrition labels of whey protein ingredients on the market, such fractionation technology has allowed the separation and concentration of whey proteins of 35-95% purity.

Since whey protein has excellent nutritive value, unique physiological benefits and diverse functionality, the use of whey protein ingredients in food applications is increasing. Whey protein ingredients are utilized to boost protein content as well as enhance flavor and texture of beverages, protein bars, baked goods, processed meats and dairy products, to name a few. Furthermore, the biological activity of whey proteins and peptides is increasing their popularity and demand as functional ingredients (Burrington, 2012a).

In 2013, the global market for whey powder, whey proteins and whey protein derivatives was worth an estimated $9.8 billion and is forecasted to reach 11.7 billion in 2017 (ADPI, 2014). The value-added ingredients, such as whey protein isolates (WPI) and WPH, have exhibited the largest growth, mostly due to the increasing demand from nutrition and health segments. In particular, nutritional and performance protein beverages, the majority of which contain whey protein ingredients, are exhibiting

profound growth, with sales topping $5.3 billion in 2013 and experiencing growth rate of 3.9% (Mintel, 2014).

### 1.3. Nutritional Value and Potential Health Benefits of Whey Protein

Consumers engaging in active lifestyles are likely to seek benefits from food and functional ingredients that can enhance physical wellbeing. Consumers are becoming aware of the nutritional and physiological advantages of whey proteins. Whey protein is a high quality, easily digestible protein, as it has a protein efficiency ratio (PER) of 3.2, and has an excellent biological value of 104, which surpasses that of other food proteins such as egg (100), casein (77), beef (80) and soy (70) (Ha and Zemel, 2003; Smithers, 2008; Pasin, 2000). Whey protein also has a Protein Digestibility Corrected Amino Acid Score (PDCAAS) of 1.14, a score that exceeds the 1.00 of soy, casein, egg whites, and beef (Pasin, 2000). Additionally, whey protein is a rich source of sulfur-containing amino acids, such as methionine and cysteine. These amino acids are important in maintaining antioxidant levels and enhancing immune function in the body by serving as precursors to glutathione, an intracellular antioxidant that protects the body against free radical damage (Walzem, 2006; Yasmin et al., 2013). Not only is whey protein a rich and balanced source of sulfur-containing amino acids, but it is also rich in all essential amino acids, particularly branched chain amino acids. Whey protein/peptides are especially beneficial for promoting muscle anabolism, weight management, satiety, and other physiological benefits.

#### 1.3.1. Muscle Anabolism

Whey protein and its essential amino acids are particularly beneficial to active individuals as they play a role in muscle anabolism. Engaging in physical activity exerts metabolic stress on the body. In order to meet the exercise-related high demand for energy, carbohydrates, fat and protein are broken down (Coyle, 2000). After exercise, the

body recovers by anabolic metabolism (Tipton and Wolfe, 1998). There are multiple means by which whey protein and its essential amino acids help promote anabolic metabolism leading to muscle recovery, and ultimately enhance athletic training and performance (Ha and Zemel, 2003). Whey protein has a high proportion (26%) of branched chain amino acids (BCAAs) such as leucine, isoleucine, and valine (Ha and Zemel, 2003; Smithers, 2008). BCAAs are the only amino acids not degraded in the liver and are instead metabolized in the skeletal muscle where they take part in protein synthesis and energy production (Layman, 2003; Walzem, 2006). Leucine in particular plays a key role in muscle repair, growth and preservation, as it acts as a dietary trigger required to initiate protein synthesis in skeletal muscle by enhancing mRNA translation (Anthony et al., 2001; Layman, 2003; Smilowitz et al., 2005; Yasmin et al., 2013). Furthermore, BCAAs help regulate lactose homeostasis in skeletal muscle and counteracts muscle protein breakdown during prolonged exercise (Yasmin et al., 2013). Overall, whey protein is an ideal protein source for athletes or active individuals seeking to maintain or increase their muscle mass.

### 1.3.2. Weight Management and Satiety

A growing body of research indicates that high-protein foods and beverages help increase satiety and play a role in regulation of food intake, body weight management and weight loss (Layman, 2003; Anderson and Moore, 2004; Halton and Hu, 2004; Weigle et al., 2005; Bowen et al., 2006; Luhovyy et al., 2007; Clifton et al., 2008; Paddon-Jones et al., 2008). While the specific effects that individual whey proteins and peptides have on reducing food intake is not fully understood yet, it is known that whey protein is better at regulating food intake compared to other proteins such as casein, carbohydrates and fat at suppressing food intake and increasing length of satiety (Anderson and Moore, 2004). This effect is greater than what can be attributed to their caloric intake alone. Also, protein digestion stimulates many physiological and metabolic pathways known to regulate food intake (Anderson and Moore, 2004). For example,

whey protein is insulinotropic, as it affects the production and activity of insulin, and whey peptides affect the renin-angiotensin aldosterone system (RAAS), which influences adipose metabolism (Gorzelniak et al., 2002; Luhovyy et al., 2007). Therefore, the use of whey protein ingredients in weight management is of current interest in industry due to the fact that it has high nutritional quality, physiologically benefits, and better ability to help regulate satiety and body weight than carbohydrates and other sources of dietary protein (Bowen et al., 2006; Luhovyy et al., 2007).

### 1.3.3. Bioactive Peptides and Physiological Effects

Whey protein possesses a number of physiological benefits due to embedded bioactive peptide sequences. The bioactive peptide sequences are active only upon the release from the polypeptide chain of the intact protein. Release of bioactive peptides often occurs upon *in-vivo* or *in-vitro* hydrolysis of the parent protein. Reported biological activities of whey peptides include gastrointestinal functions, anticarcinogenicity, antimicrobial activity, growth promotional activity, immunoactivity, and anti-hypertensive activity (Meisel et al., 1989; Walzem, 1999; Shah, 2000; Ha and Zemel, 2003; Smithers, 2008).

Antihypertensive activity is one of the most researched properties of whey derived bioactive peptides as hypertension is a prominent health issue in the U.S. Roughly 1 in 3 adults in the U.S. have hypertension, and of those, less than half have it under control (CDC, 2012). Hypertension can cause damage to blood vessels and heart, increasing risk for coronary heart disease and stroke, which are two of the leading causes of death for Americans (Kochanek et al., 2011). Overall, treatment of hypertension costs Americans over $47.5 billion each year (Heidenreich et al., 2011). This makes bioactive peptides with antihypertensive properties all the more important to research.

Whey peptides promote cardiovascular health by reducing hypertension via angiotensin I converting enzyme (ACE) inhibition (Pihlanto-Leppala, 2001). This allows

for the control of high blood pressure through dilation of blood vessels. ACE raises blood pressure by converting inactive angiotensin I hormone, derived from angiotensinogen by renin, to its active form, angiotensin II by removing the C-terminal dipeptide (Figure 1) (Pihlanto-Leppala, 2001). Studies have also shown that in addition to being a vasoconstrictor, angiotensin II is a potent proinflammatory mediator that increases oxidative stress products in the body (Dagenais and Jamali, 2005).

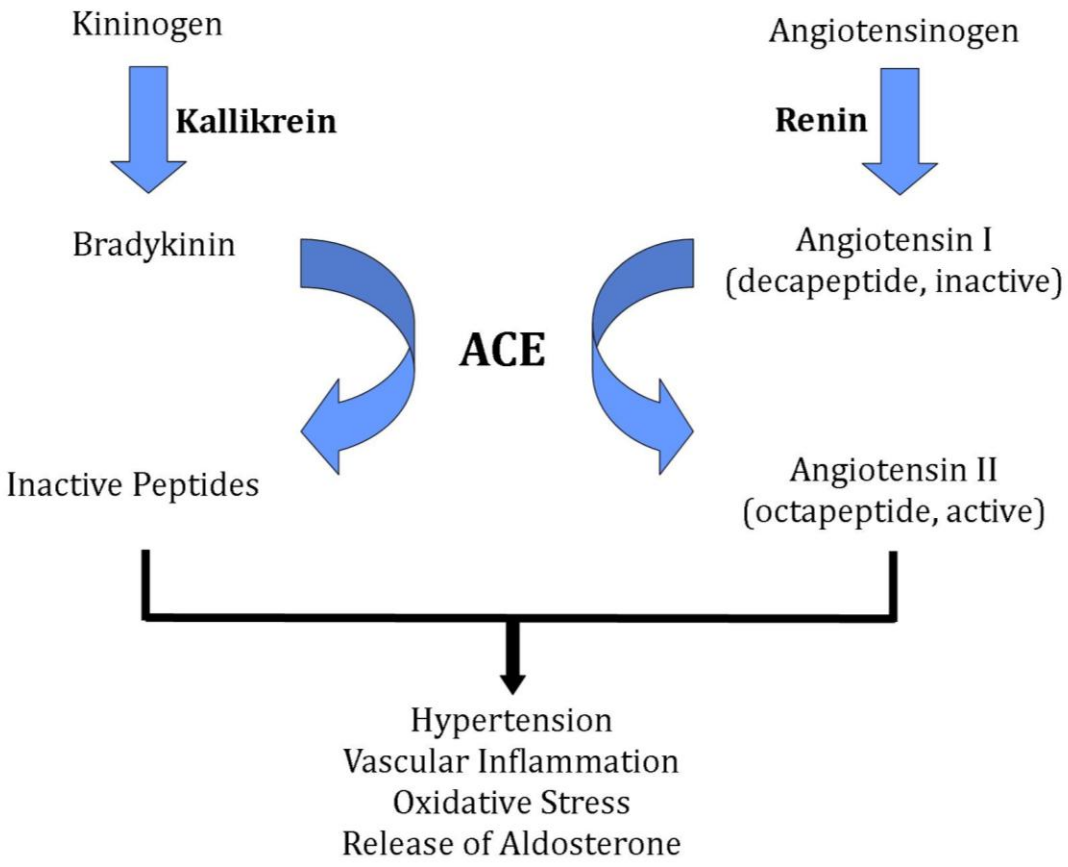


Figure 1. Overview of Angiotensin I Converting Enzyme (ACE) pathway and resulting physiological effects

ACE has many additional effects on the cardiovascular system. Notably, ACE contributes to the inactivation of the peptide bradykinin, a potent vasodilator present in

blood, and stimulates the release of aldosterone (Figure 1) from adrenal glands (Pihlanto-Leppala, 2001; Estévez et al., 2010; Hu et al., 2012). Aldosterone plays a key role in the regulation of blood pressure by increasing sodium and water retention while decreasing potassium retention (Gerdes and Harper, 2006). Dysregulation of aldosterone can lead to cardiovascular and renal disease (Figure 1) (Hu et al., 2012). Because of these functions, ACE is an ideal enzyme to inhibit when controlling blood pressure, as its inhibition can counteract these events.

Currently, there are many ACE inhibitors available commercially around the world; however, these drugs have concerning side effects, such as cough and fetal abnormalities. Thus, much research has been given to investigating the ACE inhibition properties of natural and safe bioactive peptides. A significant amount of research has shown that bioactive peptides in whey protein can inhibit ACE activity by competitive inhibition, thus preventing production of angiotensin II, the degradation of bradykinin, and thus all subsequent effects (FitzGerald and Meisel, 1999; Pihlanto-Leppala, 2001; Gerdes and Harper, 2006; Estévez et al., 2010; Pan et al., 2012). The primary whey-derived bioactive peptides showing antihypertensive properties have been found in both α -lactalbumin and β-lactoglobulin (Pihlanto-Leppala, 2001). *In-vitro* studies have found that a number of peptides originating from α-lactalbumin (α-la) f(50-53), α-la f(99-110) and dipeptides (Tyr-Gly) and (Leu-Phe) may be responsible for ACE inhibitory activity in α-la (Mullally et al., 1996; Pihlanto-Leppala, 2001). ACE inhibition activity in β-lactoglobulin (β-lg) has been attributed to many peptides originating from various regions of the β-lg chain. In general, there is no single peptide sequence that is responsible for ACE inhibitory activity. It is suggested that high ACE inhibitory activity is observed if the C terminal region of a peptide includes amino acids Trp, Tyr, and Phe or the amino acid Pro (Saito, 2008). In addition, the positive charges of Arg and/or Lys residues may further enhance inhibitory activity (Saito, 2008).

## 1.4. Whey Protein Composition

During cheese production, globular whey proteins are not coagulated by acid and are resistant to the action of rennet enzyme. Therefore, they remain behind in the liquid whey. Whey protein makes up about 20% of total milk protein and is composed primarily of 50% β-lactoglobulin (β-lg), and 20% α-lactalbumin (α-la) (Shah, 2000; Walzem, 1999). These two proteins contribute the most to whey protein functionality. The remaining fraction of whey protein consists mainly of bovine serum albumin (BSA), lactoferrin and immunoglobulins (Shah, 2000; Walzem, 1999).

### 1.4.1. β-lactoglobulin

β-lactoglobulin (Figure 2), the major component of whey, is a small globular protein that occurs as a dimer at normal milk pH 6.5. Each monomer is made up of 162 amino acid residues and occurs in several genetic variants. The variants have small differences in their amino acid sequences, but these small differences lead to considerably different physio-chemical characteristics, such as denaturation temperature, as well as effects on coagulation and renneting properties of milk (Jakob and Puhan, 1992; Farrell et al., 2004). Genetic variants A (MW 18,363 Da) and B (MW 18,277 Da) occur most frequently in bovine milk, with A expressed at higher levels than B (Farrell et al., 2004).

The secondary structure of β-lg consists of 8 antiparallel β-strands, 3-turn $\alpha$-helix on the surface and another β-strand adjoining the first strand (Papiz et al., 1986). β-lg has five cysteine residues, forming two disulfide groups (Cys 66 - Cys 160 and Cys 106 - Cys 119) and one free thiol group (Cys 121) (Papiz et al, 1986). The free thiol group becomes involved in intermolecular disulfide linkages upon protein denaturation ($\geq$ 72°C), and thus plays a substantial role in protein aggregation behavior (Havea et al., 2001). Furthermore, due to its secondary and tertiary structure, the central cavity of β-lg is hydrophobic and allows for binding of a wide range of hydrophobic and amphiphilic

molecules, such as cholesterol, fatty acids, retinol, palmitate and vitamin D (Pérez and Calvo, 1995; Wu et al., 1999; Kontopidis et al., 2004).

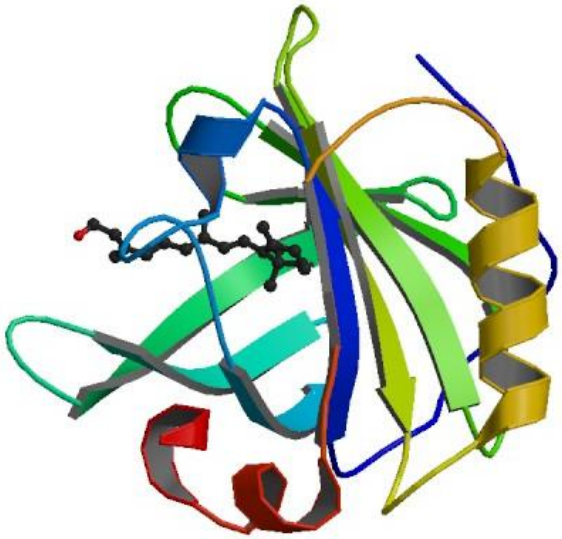

Figure 2. β-lactoglobulin structure. Interior hydrophobic cavity allows it to bind a range of ligands (RCSP Protein Data Bank, 2014).

The association of β-lg monomers depends on the pH of the system. β-lg exists as a dimer at neutral pH, and as monomers under acidic (pH < 3.5) and basic (pH > 7.5) conditions. β-lg are very stable as monomers and retain a native conformation even at high and low pH (Kuroda et al., 1996). This resistance to denaturation at extreme pH is due to excessive net charges that result in electrostatic repulsion. Between pH 3.5 and 5.2, near the isoelectric point of the protein, the electrostatic repulsion is minimized and thus the monomers aggregate to form octamers (Pelegrine and Gasparetto, 2005).

### 1.4.2. α-lactalbumin

α-lactalbumin (α-la) is a compact globular protein (Figure 3) composed of 123 amino acids and has a molecular weight of 14,147 and 14,178 Da for the predominant genetic variants A and B, respectively (Farrell et al., 2004). As described by Permyakov

and Berliner (2000), the secondary structure of α-la is comprised of two domains: a large α-helical domain made up of three major α-helices and two short α-helices, and a smaller β-sheet domain, comprised of a small three stranded antiparallel β-sheet and a short helix. Both domains are linked by a calcium binding loop. The structure is stabilized by four disulfide bridges, (Cys S6– Cys 120, Cys 61– Cys 77, Cys 73– Cys 91, and Cys 28– Cys 111).

Among the whey proteins, α-la has the lowest denaturation temperature of 62°C (Hollar et al., 1995). However, as a unique feature, α-la has a high affinity for calcium and other metal ions, stabilizing the molecule against irreversible thermal denaturation (Owusu, 1992; Albis et al., 2010). At acidic pH, presence of calcium ions stabilizes the protein and prevents aggregation at pH values as low as pH 2.9 (Pedersen et al., 2006). However, α-la undergoes substantial structural changes when it loses the bound calcium ions at acidic pHs, leaving α-la in a partially unfolded state (Pedersen et al., 2006).

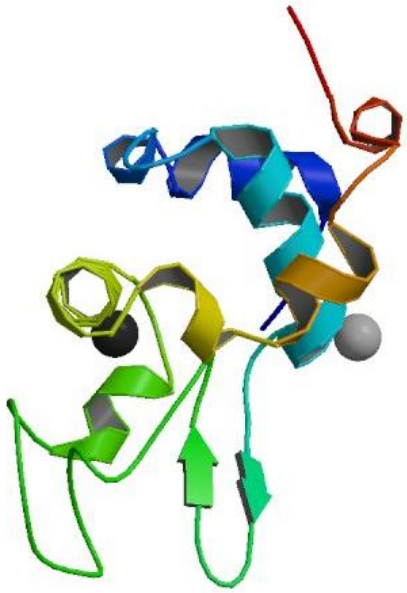

Figure 3. α-lactalbumin structure. The structure is stabilized by the presence of calcium ion and four disulfide bridges. (RCBS Protein Data Bank, 2014).

### 1.4.3. Lactoferrin

As described by Sharma et al. (2013), lactoferrin is a non-haem iron-binding glycoprotein made up of 700 amino acids and has a molecular weight of 80 kDa. Lactoferrin has two identical halves, the N-lobe and C-lobe, both of which are 40 kDa and contain one iron-binding site, are bound to each other by a short helix. Each lobe has two domains, N1 and N2 of the N-lobe, and C1 and C2 of the C-lobe. The iron-binding site is located between the two domains in each lobe and is made up of two tyrosines, one histidine, and one aspartate residue.

Lactoferrin is a multifunctional protein with antimicrobial, antiviral, antioxidant, anticarcinogenic, anti-inflammatory, and immunoactivity properties (Yalçin, 2006). The antimicrobial and antiviral properties of lactoferrin are due primarily to its iron-binding capacity, as well as its ability to bind other metal ions, such as copper, aluminum, manganese, and zinc (Lönnerdal and Iyer, 1995; Pérez and Calvo, 1995; Yalçin, 2006). The antioxidant activity of lactoferrin is attributed to its ability to scavenge free iron and prevent formation of free radicals, thus preventing iron-catalyzed oxidative damage to cells. Additionally, lactoferrin, along with other whey proteins, has been found to enhance the production of natural antioxidants, such as glutathione (Yalçin, 2006; Krissansen, 2007). Moreover, lactoferrin acts as a cancer preventative and treatment agent by causing apoptosis of certain human cancerous cells, such as in the esophagus, lung, colon and bladder (Tsuda et al., 2002; Krissansen, 2007; Chen et al., 2014), and by making chemotherapy treatments more effective (Chen et al., 2014). The anti-inflammatory property of lactoferrin, on the other hand, is mostly demonstrated in the intestinal tract as it can reduce systemic and intestinal inflammation (Lönnerdal and Iyer, 1995; Sharma et al., 2013). Finally, supplementation with lactoferrin boosts the immune system efficiency by increasing T-cell, helper T-cell and cytotoxic T-cell activation, as well as enhancing antioxidant capacity (Mulder et al., 2008).

### 1.4.4. Bovine Serum Albumin (BSA)

Bovine serum albumin (BSA) (Figure 4) is a large globular protein (66,430 Da) comprised of 583 amino acid residues (Hirayama et al., 1990). BSA has three domains termed I, II, and III, each having and each domain has 10 helical regions that are cross-linked by 17 disulfide bonds (Eigel et al., 1984). In addition, there are 9 loops (L1 to L9) that are also held together by disulfide bonds (Carter and Ho, 1994). When within the pH range of 4.5 to 8.0, the three domains assemble to form a heart shaped molecule (Carter and Ho, 1994).

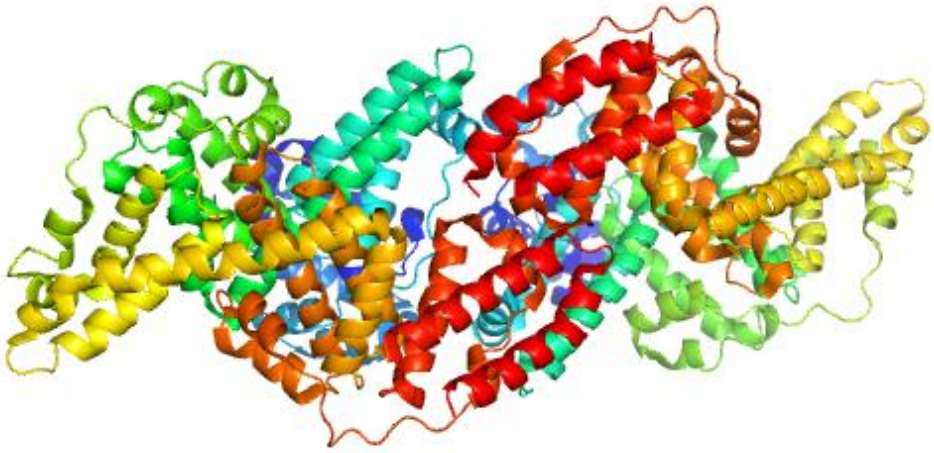

Figure 4. Bovine sera albumin (BSA) structure (New York Structural Genomics Research Consortium, 2014).

One of the many biological benefits of BSA is that it contributes antioxidant properties due to its high concentration of sulphur amino acids and glutamylcysteine, both of which are precursors for the potent antioxidant glutathione (Bounous, 2000). These properties also give BSA anti-tumor and anti-cancer activity as studies have shown BSA to inhibit tumor growth and slow the spread of cancer cells (Laursen et al. 1990). Furthermore, BSA has a good binding capacity for a number of different molecules and minerals. BSA binds water, $Ca^{2+}$, $Na^{+}$, and $K^{+}$, which allows for the regulation of the

blood colloidal osmotic pressure. BSA also has high affinity for zinc (binds 80% of all plasma zinc), and thus functions as a plasma zinc transporter (Foote et al., 1984; Cousins, 1986). Additionally, it binds fatty acids, hormones, and other small molecules to make them water soluble (Walzem et al., 2002).

### 1.4.5. Immunoglobulins

Whey contains several immunoglobulins (Ig): IgG1, IgG2, IgM, and IgA. All basic Ig molecules have a molecular mass of 160 kDa and are comprised of four polypeptide subunits, containing two heavy chains (55 kDa) and two light chains (25 kDa), to form a 'Y' shaped structure(Hurley and Theil, 2013). Disulfide bonds link heavy chains to each other, as well as bind light chains to the heavy chains (Hurley and Theil, 2013). Each Ig has a specific role and benefit to the immune system to increase defense mechanisms against bacteria, viruses, or parasites by agglutination, immobilization, and neutralization of their toxins (Bell, 2000). IgG1 is the most common, and plays a role in attacking viruses and other toxins (Bell, 2000; Hurley and Theil, 2013).

Furthermore, Ig can prove particularly useful to consumers who are immunocompromised, as bovine immunoglobulins are very similar to those found in humans, as well as many other mammalian species (Bell, 2000). They can also withstand digestion by pepsin (Watson, 1980). Much attention is currently being given to the use of immunoglobulin supplements in the treatment of AIDS and cancer. Administering immunoglobulins to such patients improved overall health and reduces secondary infections (Bell, 2000).

## 1.5. Whey Protein Ingredients

According to the U.S. Dairy Export Council (USDEC) over 1.1 million metric tons of whey and lactose products are produced worldwide each year (USDEC, 2011).

The most basic and economical dried whey ingredient is 'sweet whey powder', which contains all the constituents of fluid whey, except moisture, in the same relative proportion. The approximate composition of sweet whey powder is 11-14% protein, 63-75% lactose, 8% ash, 1% fat and 4% moisture (USDEC, 2008). The most common use of sweet whey powder is bakery, frozen dessert, and dry gravy and soup mix applications, where both the protein and significant lactose content are beneficial to product appeal and functionality. To further expand the nutritional and functional applications of whey ingredients, modern advances in processing technology such as ultrafiltration (UF), diafiltration (DF), reverse osmosis (RO), and ion-exchange chromatography (IEC) have been used to create an array of cost effective whey protein ingredients with varying amounts of protein, lactose, vitamin and mineral concentrations, as will be discussed in the following sections.

### 1.5.1. Whey Protein Concentrate (WPC) and Whey Protein Isolate (WPI)

Whey protein concentrates (WPC) have a protein concentration ranging from 35% to 85% (Bylund, 1995). The remaining portion of the concentrate is comprised of lactose, fat, and minerals. WPC is typically produced from pasteurized liquid whey using an ultrafiltration system comprised of semipermeable membranes with specific molecular cut off limits separating whey proteins from lower molecular weight components in whey, such as nonprotein nitrogen sources, lactose, vitamins and minerals (Fox and McSweeney, 1998; USDEC, 2008). The whey retentate is then subjected to reverse osmosis or passed through a multistage evaporator in order to concentrate the whey, before moving on to a spray, roller or fluid bed dryer (Fox and McSweeney, 1998; USDEC, 2008).

Whey protein isolate (WPI) has a very high protein content of at least 90%, and typically has low amounts of lactose (<1%), fat, (<1%) and ash (3%), lactoferrin, and immunoglobulins (<1%) (USDEC, 2008). WPI is produced using microfiltration or ion exchange chromatography, with optional diafiltration to produce WPI with a protein

content up to 95% (Bylund, 1995; Fox and McSweeney, 1998). As described by Sutherland (2013), one of the biggest differences between microfiltration and ion exchange chromatography manufacturing processes is found in the composition of proteins, as portions of α-lactalbumin, lactoferrin, lactoperoxidase and casein-derived glycomacropeptide (GMP) can be lost during ion exchange chromatography that are otherwise retained during microfiltration. Cation exchange chromatography processes retain very little amounts of GMP, while anion exchange and microfiltration processes retain most of the GMP. Small, but significant decreases in nonprotein components such as fat, lactose and minerals also occur in WPI produced using ion exchange chromatography compared to microfiltration.

The difference in protein, lactose, and mineral content among whey ingredients contributes to different functional properties and behavior in food applications. As the composition of WPC can be quite varied, the number of food applications for WPC is just as varied. Most often, WPC with 34% protein content is used as a product stabilizer or as a fat mimetic in dry baking mixes and dairy applications (Johnson, 2000; USDEC, 2011). In contrast, WPC with a higher protein content, such as 80%, is frequently used in nutritional beverages, dietetic foods, processed meats, soups and bakery products (USDEC, 2011). WPI can be used in a wide variety of products where functional applications such as emulsification, gelation, water-binding, solubility, whipping/foaming and viscosity are desired. But most notably, WPI is used in sports nutrition drinks, bars and instant drink mixes (3A Business Consulting, 2010).

### 1.5.2. Whey Protein Hydrolysates (WPH)

With several physiological benefits and increased functionality over WPC and WPI, much attention is being given to whey protein hydrolysates (WPH). WPH is produced by enzymatic or chemical hydrolysis to cleave peptide bonds and generate smaller peptide fractions and free amino acids, resulting in whey protein with improved functionality, digestibility and enhanced bioactivity, among other properties (Nnanna and

Wu, 2007). However, enzymatic hydrolysis is preferred over chemical hydrolysis to avoid loss of essential amino acids, such as tryptophan, as well as to avoid formation of toxic substances like lysino-alanine (Lahl and Grindstaff, 1989; Lahl and Braun, 1994). Furthermore, enzymatic hydrolysis allows for manufacturers to produce more uniform and consistent hydrolysates by precisely controlling the degree of hydrolysis, peptide composition, and molecular weight distribution (Lahl and Braun, 1994; Nnanna and Wu, 2007). There are a wide variety of enzymes that can be used to hydrolyze whey proteins, including digestive enzymes trypsin, pepsin and chymotrypsin, as well as plant derived enzymes such as papain and bromelain, and bacterial and fungal enzymes, such as subtilisin, bacillopeptidases, and aspergillopepsin (Lahl and Braun, 1994; Nnanna and Wu, 2007; Morais et al., 2014). The hydrolysis process can include a single enzymatic step, or a combination of multiple enzymes to achieve a specific desired end product. Apart from the type and specificity of the enzyme used, the degree of hydrolysis (DH), the percentage of peptides bonds cleaved by an enzyme, depends on multiple factors, such as pH, temperature and the nature of the whey protein (Morais et al., 2014).

WPH can be produced using a batch or continuous method. The traditional batch hydrolysis method is quite simple and easy to control, however it has several disadvantages, including high enzyme cost since the enzymes are used only once (Rios et al., 2004). As a solution to high production cost, enzymes can be immobilized on inert support, allowing the hydrolysis process to work continuously and the enzymes to be re-used. However, this process has its limitations as well, such as loss of enzyme activity and constrains for diffusion into the immobilized enzyme support (Sousa et al., 2004; Prieto et al., 2007). Recent advancements in technology have led to the use of continuous membrane recycle reactors. Here, soluble enzymes are mixed with the whey proteins, and the molecular weight of the hydrolyzed protein is controlled by ultrafiltration (Sousa et al., 2004; Prieto et al., 2007). Peptides of desired molecular weight can permeate the membrane, whereas the enzymes and higher molecular weight proteins are retained in the reaction tank to continue the hydrolysis process. This method allows for a cost-effective

means of production that can easily be controlled, and results in a very specific and selective hydrolysate end product (Prieto et al., 2007).

Hydrolysis of proteins has often been used to enhance the solubility, heat stability, viscosity and emulsifying and foaming properties of the protein (Fox et al., 1982; Nielsen, 2007). Whey protein solubility can be improved by 10-20% over a wide range of pH (Chobert et al., 1988; Nielsen, 2007). Hydrolysis decreases molecular size and changes conformation and strength of inter- and intramolecular interactions within the protein (Guan, 2007). The loss of secondary structure prevents heat induced structural changes, thus increasing solubility and thermal stability of the protein over a wider temperature and pH range. Furthermore, the loss of secondary structure allows for even distribution of hydrophobic and hydrophilic regions in the protein, increasing gelation, foaming and emulsification properties (Perea, Ugalde, Rodriguez & Serrat, 1993).

In terms of the nutritional properties, WPH is considered a superior alternative to WPC or WPI because the peptides are easier to digest. Studies have also shown that the amino acids and peptides in WPH are absorbed into the bloodstream, enhancing the recovery process of athletes (Boza et al., 2000; Buckley et al., 2010). Athletes recovered their full peak power and eliminated muscle soreness after just six hours of consuming WPH, compared to several days when intact whey protein was consumed (Buckley et al., 2010).

Another benefit of WPH is the presence of bioactive peptides that are released during enzymatic hydrolysis. As a result, WPH has increased health benefits attributed to several bioactivities such as antihypertensive, antioxidant, antibacterial and anticancerous properties as previously discussed (Gonzàlez-Tello et al., 1994; FitzGerald and Meisel, 1999; Pihlanto-Leppala, 2001).

Furthermore, the allergenicity of whey proteins can be reduced by destruction of epitopes upon hydrolysis (Van Beresteijn et al., 1994; Nielsen, 2007). Reducing allergenicity of whey protein benefits consumers who have developed or are at a high risk of developing allergies to milk (Van Beresteijn et al., 1994). In order to reduce or

eliminate allergenicity, extensive hydrolysis (DH ≥ 25%) has been employed (Nielsen, 2009). Extensively hydrolyzed proteins are mainly used in infant formula, as its widespread use in other food applications is limited due to a negative effect on flavor caused by the release of bitter peptides (Adler-Nissen and Olsen, 1979; Meulenbroek et al., 2014), and on the overall protein functionality (Adler-Nissen and Olsen, 1979; Lee, 1992; Lamsal et al., 2007). Much research has focused on producing a hypo-allergenic whey protein by hydrolysis, but the optimal degree of hydrolysis to eliminate an immunological response while maintaining nutritive value and functionality is unknown due to varying results. Residual allergenicity upon limited hydrolysis can differ among proteins. While some enzymes can selectively hydrolyze linear epitopes, they may result in the exposure of other epitopes buried within the three-dimensional globular structure, located in the hydrophobic domain of the protein (L'Hocine and Boye, 2007). The exposed epitopes will thus become available for IgE binding. Therefore, extensive hydrolysis that results in breakage of most peptide bonds is needed to achieve 90% or greater reduction in allergenicity.

### 1.6. Functional Properties of Whey Protein and Effects of Environmental Factors

Not only does whey protein have excellent nutritional and physiological benefits, but it has functional versatility as well. There are a number of applications for whey proteins, some of which include gelation, emulsification, increasing viscosity through water binding, adding texture, and aiding in whipping, foaming and aeration (Johnson, 2000). As a result, whey proteins have been used in a variety of food products, such as dairy products, luncheon meats, baked breads, snack foods, candies, baby formulas, protein bars and beverages (de Wit, 2001).

However, whey protein's functional properties in food applications are influenced by its structural properties, and therefore are affected by changes in the protein's globular conformations. Whey protein's conformation and molecular interactions are influenced greatly by extrinsic factors such as pH, ionic environment, concentration, presence of

lipids, and heat treatments. Whey protein has exceptional solubility over a wide range of pH, even around its isoelectric point (pI, pH 4.5-5.5), compared to other protein sources (USDEC, 2008). Whey protein's excellent solubility is due to the fact that it contains only one hydrophobic pouch on the surface of its undenatured form, resulting in low surface hydrophobicity, and thus less protein-protein interactions (Chobert et al., 1988; Wang and Ismail, 2012). Whey protein's solubility, which is greatly affected by temperature, extent of protein denaturation, and, most importantly by pH, is of primary importance due to its considerable influence on the other functional properties of whey protein, such as emulsion, foaming, gelation and whipping (Pelegrine and Gasparetto, 2005).

However, upon heating, whey protein solubility is reduced. While temperatures between 40°C and 50°C can increase protein-water interactions, and thus increase whey protein solubility, temperatures ranging from 60°C to 75°C can disrupt hydrogen and electrostatic bonds, depending on the pH and the heat stability of each whey protein (Li-Chan, 1983). Disruption of intramolecular bonds causes destabilization of secondary and tertiary protein structures and allows the protein to unfold, exposing hydrophobic and sulfhydryl groups (Li-Chan, 1983; Ye and Taylor, 2009). Hydrophobic groups participate in hydrophobic interactions while sulfhydryl groups form disulfide linkages, causing protein aggregation and possibly precipitation (Pelegrine and Gasparetto, 2005). The heat-induced aggregation via disulfide linkages is irreversible, and thus detrimental to consumer acceptance and shelf life of the product. Heating whey protein around its pI, specifically, greatly reduces whey protein solubility compared to heating at other pHs because of suppressed electrostatic repulsions and exposed hydrophobic residues, which led to increased protein-protein interaction (Pelegrine and Gasparetto, 2005). Whey proteins are more resistant to denaturation, and thus more soluble, at pHs below or above the pI due to increased net negative or positive charges (Chevalier et al., 2001b). For instance, at pH 4.0 (near the pI), β-lg starts to denature at 60°C, while at pH 2.5, denaturation of β-lg occurs above 75°C (Mulvihill and Donovan, 1987).

Whey protein solubility and thermal stability are also affected by protein concentration. A higher protein concentration increases competition for water layers and

decreases the distance between protein molecules, allowing for increased protein interaction, particularly during thermal processing when exposed hydrophobic groups and free sulfhydryl groups lead to aggregation (Pelegrine and Gasparetto, 2005). As a result, protein solubility decreases as protein concentration increases.

### 1.7. Health and Performance Food Applications of Whey Protein Ingredients

As previously mentioned, whey protein ingredients have been used in a variety of food products, but most predominantly, whey proteins have a strong position in the sports performance and nutrition markets based on the quality of proteins and functional properties they provide. A growing interest by consumers for better-for-you, high protein, more convenient ready-to-eat and ready-to-drink products has helped broaden the consumer base to include health conscious consumers in addition to its traditional core market of bodybuilders and athletes. In fact, 50% of consumers are actively trying to increase their protein intake (Raymond and Mathews, 2014). Consumers believe that consuming high protein food and beverages will help maintain healthy muscle mass, sustain energy and improve or strengthen their immune system (IFIC, 2013).

Protein beverages, specifically, are experiencing tremendous success, with beverage sales topping $521.6 million in 2013 (Mintel, 2014). Whey proteins are often the preferred source of added protein in beverage systems because of their excellent nutritional qualities, ease of digestibility, health benefits, neutral flavor profile, and great solubility over a wide range of pH (Burrington, 2012a). In addition, the ability to produce a clear, protein fortified beverage is unique only to the use of whey protein (Rittmanic, 2006).

Whey protein beverages are typically formulated either at neutral pH (typically pH 6-7) and have a milky, turbid appearance, or at acidic pH (2.8-3.5) and have high clarity and low turbidity (Rittmanic, 2006; Miller, 2007; Beecher et al., 2008a). In neutral whey protein fortified beverages, more extreme thermal treatment is required because

they must be thermally sterilized by aseptic processing or retort processing (ex. 135°C for 6.5 sec), or they must be pasteurized and refrigerated in order to be shelf stable. These processing conditions limit the whey protein concentration to less than 3% as whey proteins will precipitate or gel under these conditions unless a stabilizing system is used (Rittmanic, 2006). Such extreme thermal treatment also results in decreased sensory quality and decreased shelf life due to further protein aggregation and precipitation during storage (Rittmanic, 2006; Beecher et al., 2008a).

On the other hand, acidified whey protein beverages formulated between pH 2.8 and 3.5 have enhanced solubility and clarity, as well as increased shelf life due to milder thermal processing requirements (ex 88°C for 120 sec) and reduced protein aggregation from delayed onset of denaturation (Beecher et al., 2008b; LaClair and Etzel, 2009). Furthermore, acidic conditions allow for higher concentration of protein (~ 4%) and give beverages desirable high clarity and low turbidity (Rittmanic, 2006). However, because whey proteins have a strong buffering capacity, a large amount of acid must be added to the beverage in order to bring the pH down to 3.5 or lower. As a result, most acidified whey protein beverages fare poorly during sensory testing due to consumer dislike of sourness and astringency (Lee and Vickers, 2008; Childs and Drake, 2010). Other challenges include increasing the protein concentration to at least 4.2% while maintaining clarity for an extended period of time. A protein concentration of at least 4.2% is desired as it is the minimum percentage required by the Food and Drug Administration (FDA) to claim a "high protein beverage" (21 CFR 101.54 B) in acidified processed beverages.

Obtaining an extended shelf life for acidified whey protein beverages is also a challenge. Three to six weeks storage studies on acidified whey protein beverages with concentrations less than 4% protein and at ambient temperature revealed gradual formation of protein aggregation that resulted in undesirable turbidity, precipitation, and loss of consumer acceptability (Fox and McSweeney, 1998; LaClair and Etzel, 2009). This effect was accelerated at higher temperatures.

## 1.8. Enhancing Whey Protein Solubility and Thermal Stability in Beverages

### 1.8.1. Enzymatic Hydrolysis to Enhance Solubility and Thermal Stability

As consumers continue to become more health conscious, the demand for added value beverages is growing. Therefore, research is needed to enhance whey protein solubility and thermal stability in order to formulate beverages with more desirable pH, higher protein concentrations, and extended shelf life. Upon limited enzymatic hydrolysis (2.5-5.3%) with select proteases, whey protein solubility can be improved by 10-20% over a wide range of pH (Chobert et al., 1988; Nielsen, 2007). However, some endopeptidases, such as *B. licheniformis* protease (BLP) and Alcalase, can in fact reduce solubility by producing peptides that enhance aggregation and heat-induced gelation (Gu et al., 2011). Overall, hydrolysis using select enzymes decreases molecular size and changes conformation and strength of inter- and intramolecular interactions within the protein (Guan, 2007). The loss of secondary structure prevents heat induced structural changes, thus increasing solubility and thermal stability of the protein over a wider temperature and pH range. However, limited stability and high buffering capacity of WPH remain a concern.

In beverage applications, maintaining quality and shelf-life stability post thermal treatment is a major hurdle that hinders the use of WPH in beverages. While the thermal stability of WPH at pH 5 was reported to be slightly enhanced in relation to whey protein isolate (WPI) (Chicon, et al., 2009), WPH remained somewhat unstable under other pH conditions. Hydrophobic and disulfide interactions between whey proteins and their peptides can still occur, leading to polymerization, precipitation and separation out of the high protein beverages during manufacturing, shipping and extended storage. Furthermore, hydrolysis will result in additional free carboxyl and amine groups, thus increasing the buffering capacity of the proteins. Consequently, sourness and astringency of the high protein beverage is increased while consumer acceptability is decreased.

### 1.8.2. Maillard-Induced Glycation to Enhance Solubility and Thermal Stability

The Maillard reaction, also referred to as non-enzymatic browning, is one of the most fundamental flavor and color developing reactions in food chemistry. But this series of reactions is also gaining attention for use in protein modification to form glycoconjugates with improved functional properties. Particularly, Maillard-induced glycation has been shown to increase whey protein solubility, (Hirayama et al., 1990; Akhtar and Dickinson, 2003; Lillard et al., 2009; Hiller and Lorenzen, 2010; Sun et al., 2011; Wang and Ismail, 2012), increase thermal stability (Jiménez-Castaño et al., 2007; Wang and Ismail, 2012) and reduce heat-induced aggregation (Sun et al., 2011).

#### 1.8.2.1. The Maillard Reaction Overview

The Maillard reaction is a non-enzymatic browning reaction that involves the interaction of proteins with reducing saccharides. It is most desirable for color, flavor and aroma formation, such as in breads, syrups, meats, coffee and cocoa. However, the reaction is undesirable in instances where it causes unwanted changes in color and flavor during processing and/or storage of some food products (such as canned fruits and vegetables, UHT milk, protein bars, etc), nutritional loss of essential amino acids, such as lysine, and the formation of toxic or mutagenic compounds (Brands et al., 2000; Mauron, 1981) .

The Maillard reaction was initially described by Louis Camille Maillard during the early 1900s (Maillard, 1912) and continues to be studied today due to the many complex reaction pathways and numerous possible reaction products and intermediates. To simplify this complex series of reactions, it is commonly separated into three stages: early, advanced and final Maillard reaction (Mauron, 1981). As outlined by Hodge (1953), the early stage of the Maillard reaction is the most understood, and involves condensation of a carbonyl group of a reducing carbohydrate with an unprotonated amino group (usually that of a lysine) of a peptide or protein to give a Schiff base with the

release of water. The Schiff base cyclizes to an N-substituted aldosylamine, which then undergoes irreversible rearrangement to form an Amadori product. Since the ε-amino group of the lysine residues are the most reactive amino groups for the Maillard reaction, excessive glycation can result in nutritional quality loss due to lysine blockage (Warren and Labuza, 1977).

The advanced and finals stages of the Maillard reaction are more complex and less characterized than the early stage. In the advanced stage of the Maillard reaction, the Amadori product is degraded to deoxyosones and a number of other compounds, including aroma and flavor compounds (Mottram, 2007; Cerny, 2008). Another notable reaction pathway at this stage of the reaction includes the Strecker degradation involving α−dicarbonyl compounds, such as the deoxyosones formed in the Maillard reaction, and α-amines, giving rise to carbon dioxide and flavor compounds aminoketones and aldehydes (Mottram, 2007). Polymerization then follows, yielding formation of melanoidin by aldol condensation and heterocyclic nitrogen compounds. The Strecker degradation is also responsible for the formation of acrylamide, a known carcinogenic compound, but only at temperatures above 120°C, as is the case in fried or baked food products (Nursten, 2005, Mottram & Wedzicha, 2002). Furthermore, protein polymerization can also occur at advanced stages and reduce protein digestibility (Erbersdobler et al., 1981; Hidalgo and Zamora, 2000; Panza et al., 2010; Zhou et al., 2013). Formation of mutagenic heterocyclic aromatic amines also occur when subjected to high temperatures (> 120°C) or mild heat (< 60°C) for periods of time exceeding 80 days (Skog, Solyakov, & Arvidsson, 1998).

#### 1.8.2.2. Limited and Controlled Maillard-Induced Glycation

Limiting the Maillard reaction to the initial stage results in a glycated protein with minimal nutrition loss and enhanced functional properties. Propagation to advanced stages can be limited by optimizing reaction pH, water activity ($a_w$), weight ratio of protein to sugar, and incubation temperature and time. High temperatures promote fast

propagation of the Maillard reaction and leads to excessive browning (Martins et al., 2001). The rate of the reaction is much faster when within the optimal water activity range of 0.5-0.8 (Ames, 1990). Thus, relatively low temperatures, preferably below the denaturation temperature of the protein, short incubation times, and water activity below 0.5 will help control and limit propagation to advanced stages of the Maillard reaction.

Furthermore, the rate of the Maillard reaction can be controlled by the type and size of the carbohydrate molecules used (Chobert et al., 2006). Large carbohydrates such as dextrans (~10-43 kDa) have lower reactivity (i.e. lower reducing power) and more steric hindrance than smaller carbohydrates such as monosaccharides, limiting the advancement of the Maillard reaction and thus avoiding excessive browning and mutagenic compound formation (Jiménez-Castaño et al., 2007). The use of large carbohydrates can also preserve protein quality as large carbohydrates can shield lysine residues and prevent excessive blockage and propagation to more advanced stages in comparison to the use of shorter chain carbohydrates (Babiker et al., 1998).

#### 1.8.2.3. Mechanism of Enhanced Solubility and Thermal Stability of Whey Protein upon Glycation

In recent studies, whey protein solubility and thermal stability upon partial Maillard-induced glycation with dextran was enhanced. As reported by Wang and Ismail (2012), as compared to WPI, partially glycated whey protein (PGWP) had improved solubility and thermal stability over a wide range of pH, including the pI (4.5-5.5) of whey protein at protein concentrations up to 7% and temperatures up to 80°C. Glycation increased the denaturation temperature of whey proteins as well. The denaturation temperature of β-lg of WPI was 78°C at pH 3.4 - 5.5 and 68°C at pH 7 while the denaturation temperature of β-lg of PGWP was 84°C at the same pH ranges.

Maillard-induced glycation using dextran can improve protein solubility and thermal stability by inducing several physicochemcial/structural changes. When dextran

binds to amino groups of the protein, the net negative charges surrounding the protein increase, resulting in a shift to a more acidic pI (Chevalier et al., 2001b; Wang and Ismail, 2012). Due to the increased net negative charge, electrostatic repulsion between proteins is increased. Also, solubility is further enhanced by the increase in surface hydrophilicity due to the presence of polar polysaccharides (Wang and Ismail, 2012). Steric hindrance is increased from large dextran molecules, further reducing protein-protein interaction (Oliver et al., 2006). Overall, the combined effects of glycation on induced structural changes stabilize the protein in solution, reduce hydrophobic interactions and disulfide interchanges and slow the denaturation rate upon heating over a wide pH range (Chevalier et al., 2001b; Oliver et al., 2006; Wang and Ismail, 2012; Wang et al., 2013).

To improve protein solubility and thermal stability by glycation, while maintaining protein quality, the Maillard reaction must be controlled so that it does not propagate to advanced and final stages, where development of mutagenic compounds, off-flavors and excessive browning can occur. By using controlled reaction conditions ($a_w$, pH, temperature, protein to carbohydrate ratio) and using large carbohydrates such as dextran, the Maillard reaction can be limited to initial stages.

### 1.8.3. Enzymatic Hydrolysis combined with Maillard-Induced Glycation to Enhance Solubility and Thermal Stability

Although whey protein solubility is improved upon limited hydrolysis (<8% DH), quality and stability remain an issue. Post thermal treatment, hydrophobic and disulfide interactions between hydrolyzed whey proteins and their peptides can still occur, leading to polymerization. Whey protein hydrolysates, however, have superior physiological contributions. Limited and controlled Maillard glycation with dextran may maintain whey protein hydrolysate physiological quality while enhancing its solubility and thermal stability.

Combining the two mechanisms, hydrolysis and Maillard-induced glycation, under controlled and mild conditions may enhance solubility and thermal stability of the protein while maintaining bioactivity, digestibility and nutritional value. Currently, understanding of how these two mechanisms combined affect whey protein solubility and thermal stability is lacking, and should be explored. If the combination of these two mechanisms is successful, acidified whey protein beverages could be formulated to have a longer shelf life, as well as a protein content greater than 4.2%, which is the minimum percentage required by the FDA to claim a "high protein beverage" (21 CFR 101.54 B). In turn, the utilization and economic benefits of whey protein hydrolysates in acidified protein beverages would greatly increase, and consumer demands for a functional, high protein beverage could be fulfilled.

## 1.9. Whey Protein as a Food Allergy

### 1.9.1. Allergic Response

An allergy is referred to as a specific and reproducible immunoglobulin E (IgE) mediated immune response to inhalation, ingestion or skin contact with molecules, usually proteins (Boyce et al., 2010). The body's immune system mistakenly responds to the allergen as if it were harmful, and results in the rapid onset of symptoms within a few minutes to a few hours after contact with the allergen. Symptoms can range from mild, such as urticaria (hives), to more severe and life threatening, such as anaphylaxis. Peanuts, soybeans, fish, crustacea, milk, eggs, tree nuts, and wheat are considered the "Big Eight" as they are responsible for roughly 90% of all IgE-mediated food allergies (FDA, 2014).

As described by Larché et al. (2006), the allergic reaction occurs in two different phases: sensitization and the allergic reaction (Figure 5). The sensitization phase happens upon initial exposure to the antigen. Antigen presenting cells (APC), also referred to as

dendritic cells, recognize and process antigens, and subsequently present antigen epitopes (specific amino acid sequences) on the cell surface to naive CD4+ and CD8+ T cells, inducing differentiation of these cells into their various subtypes. The naive CD4+ T cells differentiate into allergen-specific $CD4^+$ $T_H2$ helper cells, which produce the cytokines interleukin (IL)-4, IL-5, and IL-13. As a result, secretion of these cytokines induces IgE production by allergen-specific B cells. These circulating IgE molecules then bind to the high-affinity IgE receptors (FcεRI) on the surface of mast cells in various tissues and basophils in the blood. No allergic clinical symptoms occur during the sensitization phase but, once the phase is finished, the IgE primed mast cells and basophils are now sensitive to the antigen and a second encounter with the antigen will result in an allergic response. During an allergic reaction, the epitope of the allergen cross-links IgE and FcεR on the surface of the mast cell or basophil membrane, causing cell degranulation. Release of mediators, such as histamine, leukotreines and cytokines into the blood stream and tissues during degranulation lead to the onset of symptoms within a few minutes to an hour.

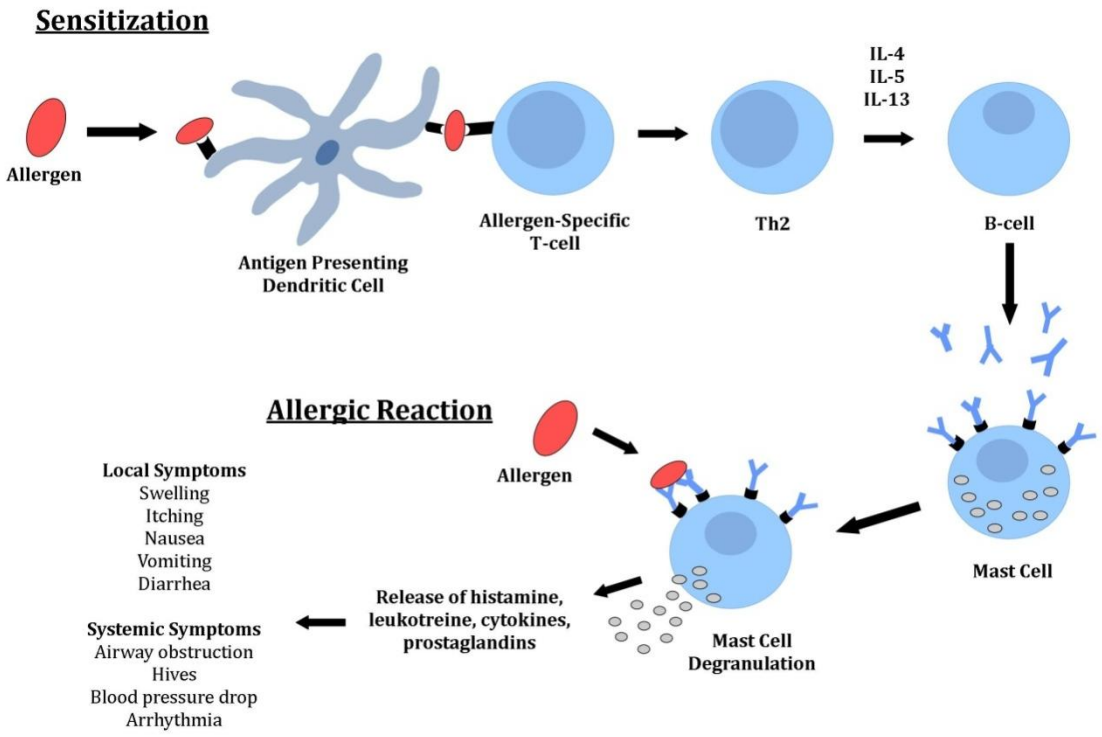


Figure 5. Overview of allergic response.

However, two antibodies must bind to the allergen in order to cross-link IgE and FcϵR (Aalberse, 2007). As a result, the allergen must have at least two IgE binding sites (epitopes). In reality, most allergens do have at least two IgE binding sites. In whey protein, alpha-lactalbumin has four IgE binding epitopes while beta-lactoglobulin was found to have seven IgE binding epitopes (Järvinen et al., 2001). These allergen epitopes can either be linear, which is simply a linear sequence of amino acids, or conformational (discontinuous), which is made up of discontinuous sections of the antigen's amino acid sequence and occur as a result of secondary and tertiary protein structure (Figure 6) (Aalberse and Crameri, 2011).

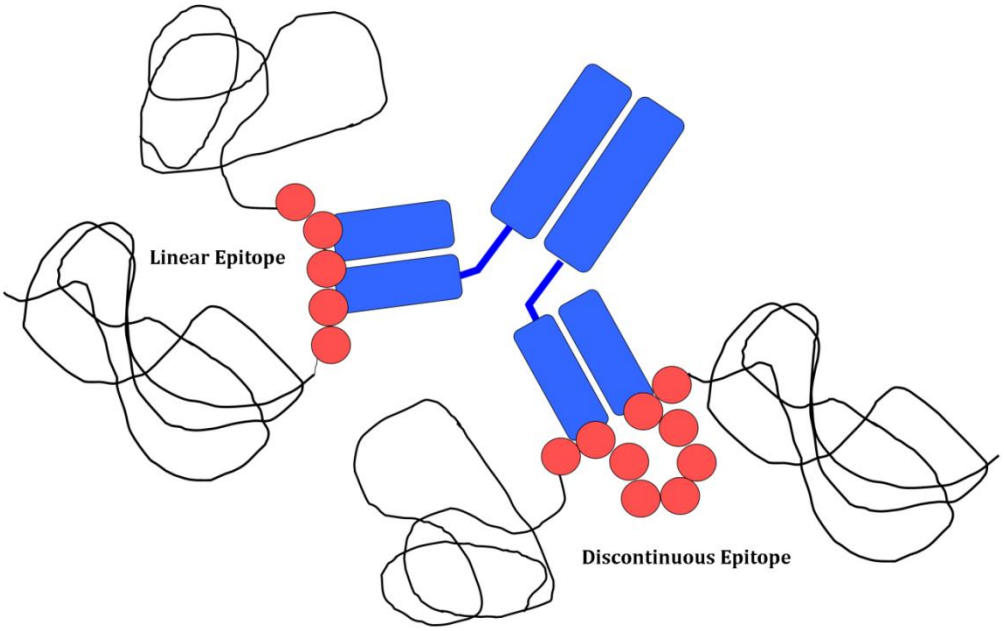


Figure 6. Linear and conformation (discontinuous) epitopes (Carbery, 2014).

### 1.9.2. Whey Allergy Prevalence

Food allergy prevalence is on the rise and is becoming of increasing concern. In the United States, it is estimated that 5.1% of children under the age of 18 years have an adverse immune response to food. There has been a 50% increase in prevalence since 1997, and the prevalence is projected to continue increasing (Branum and Lukacs, 2008; Jackson et al., 2013). In American adults, approximately 3% to 4% are affected, and also appear to have increased in prevalence (Sicherer and Sampson, 2010). Cow's milk allergy appears to be among the more prevalent food allergies in infants and children, with a rate of 2.5% (Sicherer and Sampson, 2010). This is not surprising due to increased consumption of cow's milk during a child's first couple of years. The rate of cow's milk allergy among adults is only 0.2% (Sicherer and Sampson, 2010). Although IgE antibodies for all of the main milk proteins have been reported in the sera of allergenic subjects, whey proteins β-lactoglobulin and α-lactalbumin, as well as casein (αs1-CN), are considered the dominant milk allergens (Monaci et al., 2006; Bu et al., 2013).

Furthermore, there has been an increase in the observed incidence of whey allergies over the years. This is thought to be due to the increased popularity and use of milk products by the population and the introduction of whey products and ingredients to many different food categories (Branum and Lukacs, 2008; Liu et al, 2010). It is increasingly common for the public to be exposed to whey protein in their everyday diet. This has led to increasing rates of sensitization, accidental ingestion, anaphylaxis, and even death in whey-allergic individuals (Branum and Lukacs, 2008).

### 1.9.3. Development of Allergy to Whey Protein

Milk-allergic children are often sensitized to several cow milk proteins. The major allergens in milk from cows consist of caseins and the whey proteins α-lactalbumin and β-lactoglobulin (Bu et al., 2013). The mechanism by which infants and children develop an immune response to milk proteins are thought to be due to several factors.

Development of immune response can occur upon consumption of cow's milk when there is low pepsin activity in the stomach at birth, insufficient stomach acid production, and malfunction of digestive enzymes produced in the pancreas and intestines (Monaci et al., 2006). Thus, upon consumption, milk proteins are resistant to acid and enzymatic proteolysis and are not fully digested and can remain in an intact form. (Monaci et al., 2006). Also, preliminary studies in children with cow's milk allergy demonstrated enhanced intestinal permeability due to the decreased expression of a tight junctional protein in the epithelial barrier (Fälth-Magnusson et al., 1986; Jalonen, 1991; Troncone et al., 1994). As a result of incomplete protein digestion and increased epithelial permeability, intact proteins may then pass into the intestine and stimulate a localized intestinal or a systemic immune response. The immune system recognizes the milk proteins as a foreign molecule, leading to the production of IgE specific for those milk proteins. A large portion of infants and children outgrow this milk allergy by the age of 3, while some remain allergic through adulthood (Sicherer and Sampson, 1999). Adult onset of cow's milk allergy is quite rare. It is suggested that those who outgrow their milk allergy have an allergic response to heat labile epitopes, whereas those who do not outgrow the allergy are more likely to have a response to epitopes that are less susceptible to heat (Boyano-Martínez et al., 2009).

### 1.9.4. Methods to Reduce the Allergenicity of Whey Protein

Several different protein modification techniques have been studied in order to reduce allergenicity of whey protein, including thermal treatment, enzymatic hydrolysis, and Maillard-induced glycation. Enzymatic hydrolysis and Maillard-induced glycation often target linear and sometimes conformational epitopes, while thermal treatment often targets conformational epitopes.

#### 1.9.4.1. Thermal Treatment

Thermal treatment has proven a successful method in developing whey protein ingredients with reduced allergenicity (Kilshaw et al., 1982; Norgaard et al., 1996; Paschke and Besler, 2002; Shandilya et al., 2013). Heat treatment changes protein conformation, thus conformational epitopes are disrupted. While brief heating and pasteurization of milk do not significantly reduce allergenicity, heating of cow's milk at 100°C for at least 10 minutes nearly eliminates allergic reactions to β-lg and BSA, but does not decrease reactions to caseins and α-la, which retain approximately 40 to 50% of their allergenicity (Gjesing et al., 1986; Norgaard et al., 1996; Paschke and Besler, 2002).

However, heat treatment can denature and unfold the protein, exposing linear epitopes found within the interior of the native protein. This can also create new conformational epitopes that can bind IgE. Furthermore, heat-induced changes can negatively affect functionality of the protein, as aggregation is likely to occur upon denaturation. It is sufficient to conclude that thermal treatment alone, however, cannot produce a hypoallergenic product without jeopardizing functionality, but can aid in allergenicity reduction of milk proteins.

#### 1.9.4.2. Enzymatic Hydrolysis

Another method with potential to reduce allergenicity of whey proteins is the enzymatic hydrolysis of the protein allergens (Figure 7). Enzymatic hydrolysis involves the cleavage of the peptide bond, resulting in a breakdown of the protein into smaller peptides. Thus, enzymatic hydrolysis can be used to disrupt linear epitopes and collapse conformational epitopes.

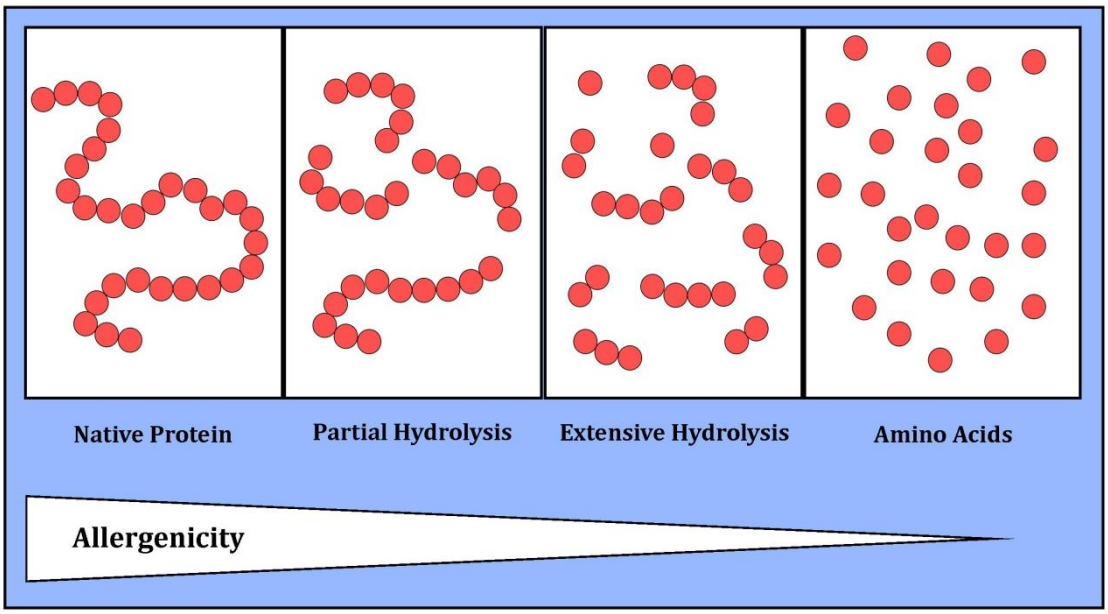


Figure 7. Reduced allergenicity of hydrolyzed protein in relation to protein size.

The resulting functionality of the hydrolyzed product greatly depends on the extent of hydrolysis and the selective destruction of epitopes (Figure 5). As with heat treatment, enzymatic hydrolysis can result in protein unfolding and thus lead to increased accessibility of hidden epitopes within the protein structures (L'Hocine and Boye, 2007). These newly accessible epitopes are then available for IgE binding. In general, studies have shown that extensive enzymatic hydrolysis (degree of hydrolysis (DH) > 25%) is very effective at reducing allergenicity of whey proteins (Nielsen, 2009). Such extensively hydrolyzed product is often used in infant formula and rarely has any other food applications due to detrimental effects on functionality. At low DH, emulsifying, whipping, foaming, or gelling capacity may be improved, however with extensive hydrolysis, these functional properties are negatively affected (Lee, 1992; Nielsen, 2007)

The release of bitter peptides upon hydrolysis is another obstacle hindering the expanded use of hydrolyzed proteins. Low molecular weight, hydrophobic bitter peptides are responsible for bitterness perception and decreased consumer acceptance, making the

ingredient undesirable in the food industry where palatability is concerned (Exl, 2001; Lamsal et al., 2007). As a result, previous research on the effect of hydrolysis of whey proteins under controlled and mild conditions to obtain a DH of 4-8% has shown moderate decrease in allergenicity while avoiding bitter peptide formations (Adler-Nissen and Olsen, 1979; Lee, 1992; Sousa et al., 2004).

#### 1.9.4.3. Maillard-Induced Glycation

Maillard-induced glycation is another protein modification technique that has been studied for reducing protein allergenicity (L'Hocine and Boye, 2007). The potential of the Maillard reaction to reduce allergenicity of a protein is promising. It has been found that the allergenicity of several protein sources such as soy (van de Lagemaat et al., 2007; Wilson et al., 2005), buckwheat (Nakamura et al., 2008), egg (Ma et al., 2013), hazelnuts (Iwan et al., 2011), peanuts (Gruber et al., 2005) and cherries (Gruber et al., 2004) has been largely reduced by glycation with a variety of carbohydrates sources. Moderate glycation resulted in only a small reduction in allergenicity, whereas a high degree of glycation has resulted in 99% reduction (Kobayashi et al., 2001; Bu et al., 2010; Li et al., 2011). The reduction in allergenicity by glycation is due to blockage of the epitope sites from sugar or polysaccharide binding or the shielding effect of the sugar or polysaccharide that prevents access of IgE to the epitopes (Usui et al., 2004; Taheri-Kafrani et al., 2009). However, with successful significant reduction in allergenicity, a high degree of glycation can be detrimental to the protein's functionality and nutritional quality for use in food applications. Additionally, previous researchers do not provide information on the protein functionality and nutritional quality, nor on the extent of progression of the reaction to advanced stages. Moreover, the conditions used, including time (days to weeks), temperature and method of glycation may not be industry feasible.

#### 1.9.4.4. Enzymatic Hydrolysis combined with Maillard-Induced Glycation

While extensive enzymatic hydrolysis can greatly reduce allergenicity of whey protein, the functionality and nutritional quality of the protein may be sacrificed. Limited hydrolysis (<8%) may reduce allergenicity of whey protein, however, not to an acceptable level. Similarly, limited and controlled Maillard glycation may reduce allergenicity of whey protein. Combining the two mechanisms, enzymatic hydrolysis and Maillard glycation under controlled and mild conditions, may have a synergistic effect on reducing allergenicity by altering potential linear and conformational epitopes, while enhancing protein functionality and maintaining physiological and nutritional quality.

## 1.10. Conclusions

Whey protein ingredients, have many nutritive, physiological, and functional benefits, and thus have a wide variety of food applications. In particular, the use of whey proteins in beverages is increasing; however hurdles such as solubility, thermal stability, and flavor prevent the expanded use of whey protein ingredients. Furthermore, with the prevalence of whey protein allergies in the United States steadily increasing, the utilization of whey protein ingredients is hindered. Limited enzymatic hydrolysis is an effective means to increase protein functionality and physiological benefits, and to reduce allergenicity of the whey protein but not to an adequate level. Extensive hydrolysis can substantially reduce allergenicity of the protein, but has negative effects on protein functionality and palatability. On the other hand, limited and controlled Maillard-induced glycation can improve protein functionality, and reduce allergenicity but not to an adequate level. Extensive glycation can substantially reduce allergenicity, but can lead to loss of nutritive value and functionality. Modifying whey protein following limited hydrolysis and controlled Maillard-induced glycation will for the first time provide information about the combined effects on whey protein solubility, thermal stability, bioactivity, and allergenicity.

# 2. Effect of Maillard-Induced Glycation on the Solubility and Thermal Stability of Whey Protein Hydrolysate

## 2.1. Overview

The physiochemical changes, solubility and thermal stability, of whey protein hydrolysate (WPH) upon partial glycation with dextran was investigated. Whey protein hydrolysate (WPH) was subjected to controlled and limited Maillard-induced glycation using dextran over 12-120 h of incubation at 60°C, 0.49 $a_w$, and a 4:1 ratio of dextran to protein. The Maillard reaction was optimized to promote sufficient glycation, while preventing propagation to advanced stages in order to minimize formation of undesirable end-products, such as melanoidins, as well as maintain nutritional quality and functionality. Based on UV-difference, fluorescence, and % free amino group loss, a 48 h incubation time was selected to produce partially glycated whey protein hydrolysate (PGWPH) with minimal propagation to advanced Maillard reaction stages and moderate free amino group loss. After incubation, ultrafiltration and hydrophobic interaction chromatography (HIC) was used to separate the nonglycated and glycated whey protein/peptides from the unreacted, free dextran. The purified had a final composition of approximately 88% protein and 12% carbohydrate. Compared to WPH, PGWPH had increased solubility and thermal stability at 5% protein concentration under acidic pH, including the pH around the isoelectric point (pI) of whey protein. Overall, this work has shown for the first time that limited and controlled Maillard glycation can be induced to produce a value added protein ingredient with potentially enhanced solubility and stability. Consequently, the utilization of biologically active WPH in acidified protein beverages would greatly increase, and consumer demands for a functional, high protein beverage could be fulfilled.

## 2.2. Introduction

Whey protein is a very popular protein source in the food industry due to its excellent nutritive and physiological properties. Whey protein is a high quality, easily digestible, complete protein. It is a rich source of sulfur-containing amino acids and branched chain amino acids, which are particularly beneficial to active individuals as they play a role in muscle repair and anabolism, satiety and weight management. Whey protein is also a source of bioactive peptides, which upon release from the parent protein contribute to several biological activities including gastrointestinal functions, anticarcinogenicity, antimicrobial activity, growth promotional activity, immunoactivity, and anti-hypertensive activity (Meisel et al., 1989; Walzem, 1999; Shah, 2000; Ha and Zemel, 2003; Smithers, 2008; Gauthier and Pouliot, 2003).

On the other hand, the physiochemical characteristics of whey protein render it versatile in functionality, namely solubility, emulsification, foaming and gelation. Accordingly, whey protein is used in a wide variety of food products and applications. In 2013, whey protein ingredients had a global market value of $9.8 billion, increasing 36% from 2011, and projected to reach $11.7 billion in 2017 (ADPI, 2014), confirming the growing awareness and interest in the functional and nutritional benefits of whey protein.

In particular, as consumers continue to become more health conscious and aware of the nutritional and physiological advantages of whey proteins, the demand for whey protein-based beverages is growing. Whey protein acidified beverages, specifically, are gaining popularity in the market. Nutritional and performance protein beverages, the majority of which contain whey protein ingredients, are exhibiting profound growth, with sales topping $5.3 billion in 2013 and experiencing a growth rate of 3.9% (Mintel, 2014). However, the market faces several challenges. While whey proteins have excellent solubility over a wide pH range and excellent clarity at acidic pH < 3.5, thermal processing and prolonged storage can result in protein aggregation and subsequent deterioration of quality (LaClair and Etzel, 2009; Dissanayake and Vasiljevic, 2009;

Burrington, 2012b). Consequently, whey protein acidified beverages available on the market have a short shelf life (typically three to six weeks) and contain at most 4% protein, which is below the minimum percentage (4.2%) required by the FDA to claim a "high protein beverage" (21 CFR 101.54 B).

In addition to having several benefits over whey protein isolate (WPI), such as presence of bioactive peptides and improved digestibility (Gonzàlez-Tello et al., 1994; FitzGerald and Meisel, 1999; Boza et al., 2000; Pihlanto-Leppala, 2001; Buckley et al., 2010), whey protein hydrolysates (WPH) may have better solubility and thermal stability. When produced under controlled hydrolysis conditions, WPH has been shown to have improved solubility over a wide range of pH (Adler-Nissen and Olsen, 1979; Chobert et al., 1988). However, maintaining quality and shelf-life stability post thermal treatment is a major hurdle that hinders the use of WPH in beverages. While the thermal stability of WPH was reported to be slightly improved compared to WPI at low protein concentrations (0.5%) (Chicón et al., 2009), thermal stability remains a hurdle for high protein concentrations (> 4%). Hydrophobic and disulfide interactions between whey proteins and their peptides can still occur, leading to polymerization, aggregation and precipitation during manufacturing, shipping and extended storage. Thus, protein content in beverages has been limited to less than 4%. Furthermore, hydrolysis will result in additional free carboxyl and amine groups, increasing the buffering capacity of the proteins. As a result, the addition of more acid to the protein beverage is required to reach an acidic pH, increasing overall sourness and astringency of the high protein beverage while decreasing consumer acceptability (Lee and Vickers, 2008).

Maillard glycation is another protein modification method that has been shown to increase whey protein solubility over a wide pH range (Hirayama et al., 1990; Akhtar and Dickinson, 2003; Lillard et al., 2009; Hiller and Lorenzen, 2010; Sun et al., 2011; Wang and Ismail, 2012), increase thermal stability (Jiménez-Castaño et al., 2007; Wang and Ismail, 2012), and reduce heat-induced aggregation (Sun et al., 2011). Maillard cross-linking between a protein and a reducing carbohydrate induces several physicochemcial/structural changes that stabilize the protein in solution, reduce

hydrophobic interactions and disulfide interchanges, and slow the denaturation rate upon heating over a wide pH range (Chevalier et al., 2001b; Oliver et al., 2006; Wang and Ismail, 2012; Wang et al., 2013). However, Maillard-induced glycation must be limited and controlled to initial stages of the reaction to limit propagation to undesired advanced stages where development of mutagenic compounds, off-flavors, excessive browning (Mauron, 1981; Brands et al., 2000; Jiménez-Castaño et al., 2007), as well as decreased digestibility (Erbersdobler et al., 1981) can occur.

It is, therefore, hypothesized that combining the two mechanisms, enzymatic hydrolysis and Maillard-induced glycation, under controlled and mild conditions, may enhance solubility and thermal stability of the protein at concentrations greater than 4.2%, while maintaining overall quality. Therefore, it is our objective to produce and characterize a partially-glycated whey protein hydrolysate following controlled and limited Maillard-induced glycation. If the combination of these two mechanisms is successful, acidified whey protein beverages could be formulated to have a longer shelf life and a protein content greater than 4.2%, allowing for a "high protein beverage" claim to be made. In turn, the utilization of biologically active WPH in acidified protein beverages would greatly increase, and consumer demands for functional, high protein beverages could be fulfilled.

## 2.3. Materials and Methods

### 2.3.1. Materials

WPI (BiPRO®, 92% protein) and WPH (BioZate®, 90% protein) were kindly provided by Davisco Foods International, Inc. (Eden Prairie, MN, USA). Dextran produced by *Leuconostoc mesenteroides* (average MW 9-11 kDa, D9260, dextrose equivalence: 2.3), o-phthaldialdehyde (OPA) (79760), L-lysine standard (56871), L-cysteine standard (52904), 5,5-dithiobis 2-nitrobenzoic acid (DNTB) (218200), 8-anilino-

1-napthalenesulfonic acid ammonium salt (ANS) (28836035), and quinine sulfate (22640) were purchased from Sigma Aldrich (St. Louis, MO, USA). Pronase enzyme from *Streptomyces griseus* (7.0 U/mg) (10165921001) was purchase from Roche Diagnostics (Indianapolis, IN, USA). Tris-tricine Criterion™ precast peptide gels (345-0068), tricine sample buffer (161-0739), Precision Plus Protein™ Dual Xtra prestained molecular weight ladder (161-D377), and concentrated tris-tricine-sodium dodecyl sulfate running buffer (161-0744) were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Pierce™ bicinchoninic acid (BCA) protein assay kit (23227), Pierce™ glycoprotein staining kit (PI-24562), bovine serum albumin liquid standard (23210), and Snakeskin™ dialysis tubing (3.5kDa MWCO) (38035) were purchased from Thermo Scientific (Waltham, MA, USA). Amicon Ultra-15 centrifugal filter units with Ultracel-3 membranes were purchased from EMD Millipore (UFC900324) (Billeria MA, USA). Opaque, black (Costar® 3916); opaque, white (Falcon® 35-3296); and UV-specific (Costar® 3635) 96-well polystyrene microplates were purchased from Corning, Inc. (Corning, NY, USA). High Performance phenyl sepharose media (17-1082-01) and XK-16/20 column were purchased from GE® Healthcare (Little Chalfont, UK). Celltreat® 0.45 µm filters were purchased from Celltreat Scientific Products (Shirley, MA, USA). All other reagent grade chemicals were purchased from Fisher Scientific (Waltham, MA, USA) or Sigma-Aldrich (St. Louis, MO, USA)

### 2.3.2. Controlled Maillard-Induced Glycation of Whey Protein Hydrolysate

Best incubation conditions, such as temperature, water activity ($a_w$), and protein-to-dextran ratio for optimal production of Amadori compounds (glycated protein) and minimal browning were previously determined for WPI by Wang & Ismail (2012). To ensure the same incubation conditions were optimal for WPH, WPH was mixed with dextran in 1:4 ratio (w/w), dissolved in phosphate buffer (0.01 M, pH 7) and lyophilized. The lyophilized powder was then incubated in triplicate in desiccators equilibrated at 60°C and 0.49 $a_w$ using saturated sodium bromide slurries, and withdrawn after 12, 24,

36, 48, 60, 72, 84, 96, 108, and 120 h of storage. Dextran was chosen as the reducing carbohydrate due to the fact that the Maillard reaction progresses at a slower rate using longer chain reducing saccharides compared to smaller reducing sugars (Jiménez-Castaño et al., 2007), thus allowing for better control of the reaction. The protein-to-dextran ratio and $a_w$ were chosen based on previous work by Wang and Ismail (2012). The $a_w$ was set at 0.49, which is below the optimum for fast propagation of Maillard reaction (Jiménez-Castaño et al., 2005). Incubation temperature was set at 60°C to limit protein denaturation and aggregation. As a control, WPH was prepared in 0.01 M phosphate buffer without the addition of dextran, lyophilized, and incubated separately under the same conditions for all time points. Upon removal from the desiccators at the predetermined time points, samples were stored at -20°C until analysis.

### 2.3.3. Estimation of Maillard Glycation Extent of WPH Incubated with Dextran

To estimate the extent of glycation that occurred over the course of the 12-120 h of incubation, and to aid in selecting an optimum incubation time for the production of glycated WPH for further study, a variety of analyses were carried out, including estimation of Amadori compounds and browning following UV-VIS difference spectroscopy, estimation of fluorescent Maillard compounds using fluorescence spectroscopy, estimation of amino group loss following o-phthaldialdehyde (OPA) method, and visualization of protein/peptide molecular weight distribution following sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

#### 2.3.3.1. Monitoring of Amadori Compound Formation and Browning

As indicators of propagation to advanced stages of the Maillard reaction, Amadori compound formation and browning were monitored at each incubation time point following UV-Visible difference spectroscopy as outlined by Wang and Ismail (2012),

with modifications. Samples and controls (incubated WPH with and without dextran), as well as non-incubated WPH, in triplicate, were dissolved in distilled, deionized water (DDW) to make a 0.2% protein solution (w/v) and centrifuged (13,800 x g for 10 min). The absorbance of the supernatant (200 µL) was measured at 280 nm to correct for protein content. The difference UV absorption (DUV) at 304 nm of the samples and the controls was measured, and served as an indication of Amadori compound formation. Difference in absorption at 420 nm between samples and controls was also monitored, and served as an indication of browning. The change in Amadori compound formation and browning over time was determined by comparing WPH incubated with or without dextran to non-incubated controls.

#### 2.3.3.2. Determination of Fluorescent Compounds

The change in fluorescent compound formation in WPH incubated with and without dextran over time was monitored as described by Rao et al. (2012), with modifications. WPH incubated with dextran, WPH incubated without dextran, and non-incubated controls were analyzed in triplicate. For each, a 1.0% protein solution (w/v) was prepared in DDW. An aliquot (0.178 mL) of pronase solution (20 U/mL pronase in 50 mM tris-HCl, pH 7.2), made fresh daily, was added to each sample, which was then incubated on a carousel mixer for 50 min at room temperature to ensure complete digestion. After digestion, the samples were centrifuged at 15,682 x g for 15 min, and the supernatant was removed and diluted 1:1 (v/v) with phosphate buffered saline solution (20 mM phosphate buffer, 15 mM NaCl, pH 7.0). An aliquot of 200 µL of each sample was placed into the wells of a black, opaque microplate, and the fluorescence intensity (FI) was measured at an excitation of 360 nm (bandwidth 40 nm) and emission of 460 nm (bandwidth 30 nm). Readings were corrected for protein content using the BCA assay kit, following the manufacturer's instructions. The fluorescence intensity (FI) of samples was determined by first correcting for the protein content of the sample. Then the FI values of all incubated samples were adjusted by subtracting the FI values of their respective non-

incubated controls, which served as blanks. The FI values were then corrected to the FI of 5 ppm quinine sulfate solution which was prepared fresh daily by diluting quinine sulfate sulfuric acid solution (100 μg/mL quinine sulfate in 50 mM sulfuric acid) to 5 ppm with phosphate buffered saline solution, as in **Equation 1**.

**Equation 1:**

$$\%\ Fluorescence\ Intensity\ (FI)/\ g\ protein\ = \left( \frac{\frac{Emi_{sample}}{g\ Protein} - \frac{Emi_{control}}{g\ Protein}}{Emi_{5\ ppm\ Quinine\ Sulfate}} \right)$$

Where:

Emi = emission reading at 460 nm (bandwidth 30 nm)

#### 2.3.3.3. Loss of Free Amino Groups

The loss of free amino groups of the WPH incubated with dextran was assessed by the *O*-Phthaladehyde method as outlined by Goodno et al. (1981) and Rao et al. (2012) using a lysine standard, with modifications. WPH incubated with and without dextran, and non-incubated WPH samples were prepared in triplicate by dissolving in 1% sodium dodecyl sulfate (SDS) (w/v) to make a 0.2% protein solution. Samples were then vortexed and centrifuged at 15,682 x g for 10 min. Supernatant was removed and diluted 10x for the OPA method and subsequently diluted 2x more for a total of 20x dilution from the original solution for protein analysis using the Thermo Scientific™ Pierce™ BCA assay kit, following the manufacturer's instructions. Standards of L-lysine (0-200 μg/mL) were prepared in 1% SDS (w/v) and used to construct a standard curve. 1% SDS (w/v) served as the blank.

The OPA reagent was made by dissolving 80 mg of OPA (dissolved in 2 mL 200 proof ethanol) and 200 μL of β-mercaptoethanol (BME) in 0.1 M sodium tetraborate with 0.5% (g/mL) SDS, to make up a volume of 100 mL. The OPA reagent was shielded from

light and used within 2 h of preparation. Each sample, standard, and blank (50 μL/well) was added into a 96-well clear, flat bottom polystyrene plate specific for UV readings (Corning, Inc., Corning, NY, USA). Subsequently, the OPA reagent (200 μL/well) was added and the microplate was immediately placed in the microplate reader (BioTek Synergy HT, BioTek Instruments, Winooski, VT, USA). The plate was briefly shaken and held for 2 min at room temperature prior to reading the absorbance at 340 nm. Free amino group concentration (mg/mL) in each sample was adjusted for the protein content (mg/mL) of each sample to obtain % free amino groups per protein content. Remaining free amino group content over time was determined using **Equation 2.**

**Equation 2:**

$$\%\ \text{Remaining Free Amino Groups} = 100 - \frac{100 * (\%\ \text{free amino groups}_0 - \%\ \text{free amino groups}_{incubated})}{\%\ \text{free amino groups}_0}$$

### 2.3.3.4. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The change in molecular size of proteins/peptides in WPH incubated with dextran over time was monitored by running an SDS-PAGE using tris-tricine peptide gels as outlined by Laemmli (1970), and Schägger and von Jagow (1987), with modifications. WPH incubated with dextran and non-incubated controls were analyzed in triplicate. For each, a 0.02% protein solution (w/v) was prepared in DDW before being diluted 1:1 (v/v) with tricine sample buffer containing SDS and 2% (w/w) β-mercaptoethanol. All samples were then heated in boiling water for 5 min and allowed to cool to room temperature. A 10 μL aliquot of each sample was loaded onto a precast 10-20% gradient tris-tricine peptide gel, along with a 10 μL aliquot of a prestained molecular weight ladder. All gels were electrophoresed with tris-tricine-SDS buffer at 4°C with a voltage of 125 for 3-4 h. Gels were then stained using either Coomassie blue stain or the Pierce™ Glycoprotein Staining Kit.

#### 2.3.3.4.1. Coomassie Blue Staining of Polyacrylamide Gels

Coomassie blue staining was carried out by first immersing gels in Coomassie blue stain solution (45% methanol (v/v), 10% glacial acetic acid (v/v), and 0.3% Brilliant Blue R250 (w/v) for 1 h while shaking. Gels were destained by repeated rinsing with a destaining solution (85% DDW (v/v), 10% glacial acetic acid (v/v), 5% methanol (v/v)). Visualization of the gel was done using a Canon T3i camera with the gel positioned over a fluorescent back-lit platform for optimal resolution.

#### 2.3.3.4.2. Glycoprotein Staining of Polyacrylamide Gels

Glycoprotein staining was carried out on a second set of gels with a Pierce™ Glycoprotein Staining Kit. Staining and destaining was performed according to the manufacturer's instructions. Visualization of gels was done as outlined in Section 2.3.3.4.1.

### 2.3.4. Separation of Partially Glycated Whey Protein Hydrolysate (PGWPH) from Free Dextran

Based on the results of estimated glycation and loss of free amino groups, 48 h was chosen as the optimal incubation time for WPH and dextran to form partially glycated whey proteins and peptides with minimal propagation to intermediate and advanced stages of the Maillard reaction, as indicated by fluorescent compound formation and browning, as will be discussed later in the results section. In order to prevent interferences during further testing and to avoid further Maillard glycation during storage, any free, unreacted dextran was separated from the glycated and non-glycated whey protein and peptides, hereafter referred to as partially glycated whey protein

hydrolysate (PGWPH), using ultrafiltration and hydrophobic interaction chromatography (HIC) based on the work by Wang and Ismail (2012) and Ruud (2015). Wang and Ismail (2012) used HIC to separate free dextran from glycated and non-glycated WPI. This method was further explored and expanded upon to separate free dextran from glycated and non-glycated whey protein hydrolysates, as described by Ruud (2015). Modifications included scaled-up chromatographic conditions to increase the efficiency and throughput of the free dextran separation protocol, as well as the addition of an ultrafiltration step in order to increase retention of valuable, low molecular weight peptides. Short chain non-hydrophobic peptides were being lost during the elution of the free dextran following HIC separation. Therefore, by ultrafiltering the incubated protein/dextran sample using 3 kDa centrifugal filtration devices before HIC was carried out, peptides were collected in the permeate, stored, and later recombined with the glycated and non-glycated whey protein/peptide separated by HIC.

Once ultrafiltration retentates and permeates were bulked and lyophilized separately, the bulked retentate was subjected to HIC to separate out free dextran, as described in Wang and Ismail (2012), with some modifications. Briefly, a Shimadzu Chromatograph system, equipped with a UV detector (Shimadzu Corp., Kyoto, Japan), and an XK 16/20 HIC column, 20 cm x 16 mm (GE Healthcare®), which was packed with high performance phenyl sepharose media and equilibrated with 2 M ammonium sulfate, pH 7, was used. Incubated protein/dextran sample was dissolved in DDW (4% protein, w/v), and a 4 mL aliquot of the solution was injected onto the column at a flow rate of 3 mL/min. Elution of the protein/peptides was monitored at 220 nm, using EZStart™ software (Shimadzu, Kyoto, Japan). Free dextran was eluted using 72 mL of 2M ammonium sulfate, and PGWPH was subsequently eluted using 120 mL DDW. Ammonium sulfate concentration and elution volumes were experimentally adjusted to achieve best possible separation of free dextran and recovery of retentate PGWPH. To prevent column fouling, the column was washed with 300 mL of 0.1N NaOH after every third injection in order to elute strongly bound proteins.

Dextran content of the collected fractions at intervals of 3 min were determined experimentally using the AOAC phenol-sulfuric acid method (Official Method 988.12, AOAC International, 1988), with modifications. Briefly, fractions were diluted with DDW to reach a carbohydrate concentration of 50 μg/mL. An aliquot (1mL) of diluted sample was taken, and 25 μL of 80% (v/v) phenol and 2.5 mL concentrated sulfuric acid were added. All samples were vortexed for 5 s and allowed to stand for 10 min to cool to room temperature. Glucose standards with concentrations ranging from 0-100 μg/mL were prepared in a similar fashion to construct a standard curve. The absorbance of each standard and diluted fraction was measured at 490 nm using a spectrophotometer.

Upon HIC separation, eluted PGWPH was collected, neutralized to pH 7, and immediately dialyzed using 3.5 kDa MWCO dialysis tubing. Desalted PGWPH solution was lyophilized, recombined with peptide permeates, pulverized to a powder consistency, and stored at -20°C until further analysis.

### 2.3.5. Analysis of Protein and Carbohydrate Content

To determine the protein content of PGWPH before and after HIC separation, the Dumas nitrogen combustion method (AOAC # 968.06, AOAC International, 1998) was followed using a Nitrogen Analyzer (LECO® TruSpecN™, St. Joseph, MI, USA). A nitrogen conversion factor of 6.38 was used. Dextran content of HIC separated PGWPH was determined using the AOAC phenol-sulfuric acid method, with modifications. Briefly, a 0.01% protein solution (w/v) of PGWPH was prepared in DDW, in duplicate. Analysis was completed as outlined under section 2.3.4.

### 2.3.6. Functionality of PGWPH

Changes in protein solubility and thermal stability of WPH upon glycation were determined. Surface hydrophobicity and total sulfhydryl groups of PGWPH compared to

WPH and WPI were also assessed in order to understand the physio-chemical changes that occurred upon glycation and subsequent heat treatment. SDS-PAGE was also run in order to visualize and better understand the protein-protein interactions that occurred upon heating.

### 2.3.6.1. Quantitative Determination of Protein Solubility

Solutions (1 mL in DDW) of WPH and PGWPH were prepared in 1.5 mL microcentrifuge tubes, in triplicate, at 5% protein concentration (w/v). To simulate the range of pH used when formulating acidic whey protein beverages and to test the effect of heating at the isoelectric point, solutions were adjusted to either pH 3.4, 4.5, or 5.5 using aliquots of 3M HCl and 3M NaOH and an Orion™ ROSS Ultra™ pH Electrode (Thermo Scientific, Waltham, MA, USA). Solutions at each protein concentration and pH were either left at room temperature or subjected to heating at 80ºC for 30 min in a water bath. The protein content of each solution was determined by DUMAS using LECO® TruSpecN™ nitrogen analyzer as described in section 2.3.5. All samples were centrifuged (13,000 x g for 10 min at 23ºC), and protein content in the supernatant was determined by DUMAS. To determine the protein solubility at each time point for each sample type, **Equation 3** was used.

**Equation 3:**

$$\%\ \text{protein solubility} = \frac{(\text{original protein } \% - \text{supernatant protein } \%)}{\text{original protein } \%}$$

### 2.3.6.2. Determination of Surface Sulfhydryl Groups

Surface sulfhydryl groups of PGWPH and WPH was determined as outlined by Sava and Planken (2005), with some modifications. WPI, WPH and PGWPH samples

(5% protein w/v) were prepared in triplicate using DDW. An aliquot (1 mL) of each sample was subjected to heat treatment (75°C) by placing in a heated water bath for 0, 10, 20, 30, 40, 50 and 60 min. All samples were diluted to 0.1% protein (w/v) using Tris buffer (0.086 M Tris, 0.09 M glycine, and 4mM $Na_2EDTA$; pH=8). 10 µL of 5,5'-dithiobis-(2-nitrobenzoic acid (DTNB) (4 mg/mL in tris buffer) was added to 200 µL of sample in a UV Costar 96 well microplate, and the absorbance was measured at 412 nm against a reagent blank after 15 min holding time at 23ºC in a microplate reader (BioTek Synergy HT, BioTek Instruments, Winooski, VT, USA). A calibration curve was prepared using standard cysteine solutions (10, 20, 40, 60, 80, & 100 µM) in Tris buffer.

#### 2.3.6.3. Determination of Surface Hydrophobicity

The change in surface hydrophobicity of PGWPH and WPH was determined spectrofluorometrically as described by Kato and Nakai (1980) and Sava and Planken (2005), with modifications. WPI, WPH and PGWPH samples (5% protein w/v) were prepared in triplicate using DDW. An aliquot (1 mL) of each sample was subjected to heat treatment (75°C) by placing in a heated water bath for up to 1 h, with aliquots drawn every 10 min. Sample aliquots were then diluted to concentrations ranging from 0.005 - 0.050% protein (w/v) using 0.017 M: 0.165 M citric acid/sodium phosphate (pH 7). 8-anilino-1-napthalenesulfonic acid ammonium salt (ANS) probe solution (10 µL, 25.3 µg/mL) was added to 200 µL of the diluted samples in a white opaque 96 well plate. The relative fluorescence index (RFI) was measured at excitation and emission wavelengths of 400/30 (wavelength/bandwidth) and 460/40 nm and 25 gain after 15 min holding in the dark at room temperature using a microplate reader (BioTek Synergy HT, BioTek Instruments, Winooski, VT, USA). The RFI of each protein dilution containing no ANS probe was subtracted from that of corresponding protein solution with ANS to obtain net RFI. The slope of the net RFIs at each heat treatment time point plotted against % protein concentration was used as an index of the protein surface hydrophobicity.

#### 2.3.6.4. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out to visualize the protein distribution in the non-heated and heated WPH and PGWPH samples prepared as outlined in sections 2.3.6.2 and 2.3.6.3. Heated and non-heated 5% protein solutions were diluted to 2 mg/mL protein concentration, mixed in 1:1 ratio with Laemmli buffer and boiled for 5 min. For reducing conditions, β-mercaptoethanol was added (5%, v/v). An aliquot (10 μL) of each sample was loaded onto a precast 10-20% gradient tris-tricine peptide gel, along with a 10 μL aliquot of a prestained molecular weight ladder. All gels were electrophoresed with tris-tricine-SDS buffer at 4°C with a voltage of 125 for 3-4 h. Gels were then stained using Coomassie Brilliant Blue for 1 h followed by de-staining as outlined in sections 2.3.3.4.

### 2.3.7. Statistical Analysis

Analysis of variance (ANOVA) was carried out using IBM SPSS Statistics software version 22.0 for Windows (SPSS, Inc., Chicago, IL, USA). Significant differences among the means of different treatments were determined when a factor effect or an interaction was found to be significant ($P \leq 0.05$) using the Tukey-Kramer multiple means comparison test. ANOVA tables for Chapter 2 can be found in Appendix F (Tables 8-14).

## 2.4. Results and Discussion

### 2.4.1. Extent of Maillard-Induced Glycation of WPH Incubated with Dextran

#### 2.4.1.1. Amadori Compound and Melanoidin Formation

Since Amadori compounds are the first stable Maillard reaction intermediate, the extent of Maillard-induced glycation of WPH with dextran was estimated by the quantification of Amadori compound using differential UV (DUV) absorbance at 304 nm (Feather et al., 1996). The resulting differences in absorbance at 304 nm of WPH incubated with and without dextran confirmed that glycation did occur during incubation at 60°C and 0.49 $a_w$ (Figure 8). The significant difference in absorbance between non-incubated 0 h and 12 h incubated WPH with dextran indicated that Maillard-induced glycation was initiated within the first 12 h of incubation. Formation of Amadori compounds continued to occur as incubation time increased through 108 h, as indicated by significant increases in absorbance, suggesting further glycation of WPH with dextran. After 108 h, the reaction seemed to plateau, suggesting an equilibrium between new Amadori compound formation and loss of Amadori compounds as a result of conversion to intermediate and advanced glycation end-products. It should be noted that WPH incubated with dextran at 72 h had a higher Amadori compound formation than 84 or 96 h incubation time points. This is likely due to the fact that samples for each time point were incubated in separate desiccators. Slight differences in environmental conditions could have occurred between desiccators, such as water activity and temperature fluctuation, leading to increased rates of Amadori compound formation, as seen at 72 h.

These results were similar to those observed in a recent study by researchers Wang and Ismail (2012), where Maillard-induced glycation of WPI was carried out using 9-10 kDa dextran under the same conditions as this study. However, Wang and Ismail (2012) observed a much slower rate of Amadori compound formation using WPI. This is likely due to the increased number of available free amino groups in WPH compared to

WPI, increased molecular mobility, and increased reactivity of protein hydrolysates. As a result, the Maillard reaction more readily took place using WPH compared to WPI under the same reaction conditions. Additionally, Zhu et al. (2008) assessed browning of dextran and WPI in solution at protein concentrations of 0-10% (w/v) under similar temperature and pH conditions. After 24 h of incubation, the extent of Amadori compound formation was considerably higher than that observed from the dry glycation carried out in this study, suggesting dry glycation may offer better control of the progress of Maillard reaction.

Amadori compound formation was also observed in the controls. This observation was likely due to two reasons; 1) the presence of a minute amount of lactose in the WPH used (< 1%, according to manufacturer's specifications), which can participate in the Maillard reaction with the whey protein, and thus lead to the formation of Amadori compounds, and 2) the higher number of available free amino groups, which increased the reactivity of WPH, as previously discussed.

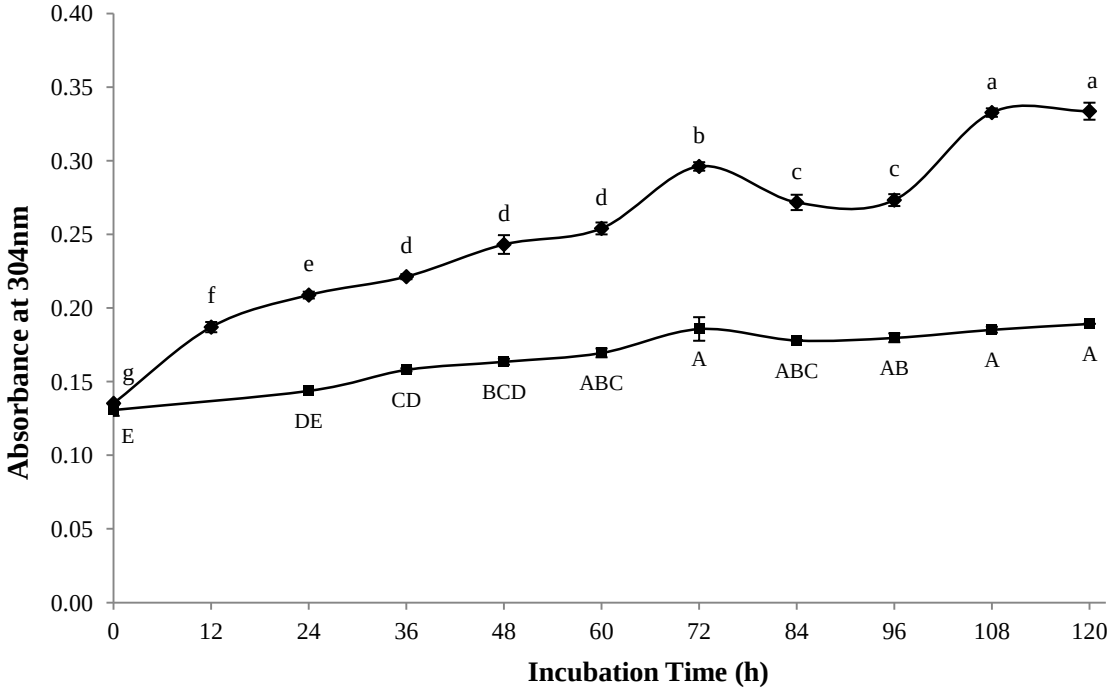


Figure 8. Amadori compound formation in samples of whey protein hydrolysate (WPH) incubated with dextran (◆) and control WPH incubated without dextran (■) at 60°C, 0.49 $a_w$, for 0-120 h, as determined by UV-Visible difference spectroscopy at 304 nm. Error bars represent standard errors (n=3). Different lower case and upper case letters above and below the shapes indicate significant differences between different time points for samples according to Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

To monitor the propagation of the Maillard reaction to advanced stages, browning due to the formation of melanoidins, which is one of the many advanced stage glycation end-products, was monitored using absorbance at 420 nm (Martins et al., 2000). Results indicated that browning significantly increased over the 120 h incubation time period (Figure 19 in Appendix A). However, the extent of browning is considered minimal as the absorbance at 420 nm remained below 0.025 through 120 h of incubation. These results are similar with other researchers' findings. Wang and Ismail (2012) observed 420 nm absorbance below 0.020 for WPI incubated with dextran at 60°C for up to 140 h. Corzo-Martinez et al. (2008) observed that the absorbance at 420 nm of β-lactoglobulin

incubated with galactose at 50°C and 0.44 $a_w$ up to 60 h of incubation remained below 0.020. Overall, the results in this study are consistent with a limited formation of advanced glycation end products.

#### 2.4.1.2. Fluorescence Compound Formation

To further understand the progression of the Maillard reaction in the protein/dextran samples, the formation of fluorescent compounds was used as an indicator of propagation beyond initial stages of the Maillard reaction. After Amadori rearrangement in the initial stages, intermediate compounds will undergo further reactions, such as dehydration and fission, to form fluorescent colorless intermediates (Bastos et al., 2012b). These intermediates are considered precursors of brown pigments, and thus allow monitoring of progression beyond initial stages of the Maillard reaction before any visual changes have occurred, such as melanoidin production in advanced stages of the reaction (Burton and McWeeny, 1963; Baisier and Labuza, 1992; Morales Navas et al., 1995; Bastos et al., 2012a).

The % fluorescence intensity of WPH incubated with dextran significantly increased over time (Figure 9), indicating fluorescence compound formation and, hence, propagation to intermediate reaction stages. The increase in % fluorescence intensity increased considerably during the 96 to 120 h incubation period, indicating progression to intermediate stage of the Maillard reaction was more pronounced during this time period. Similar to the pattern observed in Amadori compound formation, the values observed of WPH incubated with and without dextran for 72 h were higher than that of the 84 and 96h time points, suggesting that a greater amount of Maillard reaction occurred in these samples due to slightly different environmental conditions during incubation, as discussed earlier. Control WPH incubated without dextran also exhibited some fluorescence activity over time, indicating the occurrence of the Maillard reaction. This is in agreement with the observations of UV-VIS difference spectroscopy and likely due to the presence of minute amounts of lactose, as previously discussed.

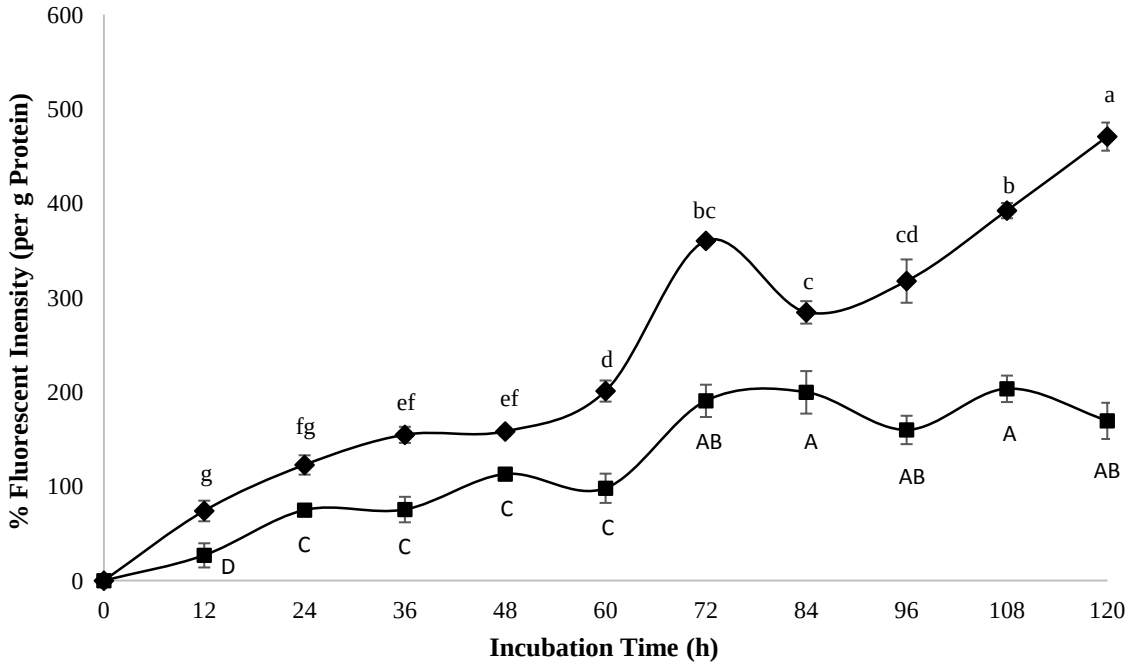


Figure 9. Fluorescent compound formation for whey protein hydrolysate (WPH) incubated with dextran (◆) and control WPH incubated without dextran (■) at 60°C for 0-120 h at 0.49 $a_w$ as determined by fluorescent intensity quantification. Error bars represent standard errors (n=3). Different uppercase and lowercase letters above or below the shapes indicate significant differences between different time points according to the Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

It should be noted that fluorescent compounds may originate from chemical reactions other than the Maillard reaction, such as lipid oxidation (Hidalgo and Zamora, 2000; Castilho et al., 1994). However, the WPH used in this study had minimal lipid content (less than 0.5%) (by manufacturer's specification) and the mild conditions used in this study make for the progression of other possible browning reactions very difficult. Thus extent of fluorescent compound formation is attributed to Maillard-glycation alone.

#### 2.4.1.3. Loss of Free Amino Groups

Since the Maillard reaction involves a reducing sugar and a free amino group (mainly the ε-amino group of lysine, but also the α-amino groups of terminal amino acids), the extent of glycation can also be assessed by determining the extent of amine blockage. As determined by the OPA method, the extent of free amino group loss was moderate. After 12 h of incubation, a significant decrease in free amine groups (15.9%) was noted for WPH, confirming that the Maillard-glycation was initiated within the first 12 h (Figure 10). Maillard-glycation continued thereafter, as the % free amine groups remaining continued to decrease to 69% as incubation time reached 120 h. These results are in accordance with the UV-VIS difference spectroscopy data discussed earlier, which also indicated that Maillard-glycation was initiated within the first 12 h of incubation and continued thereafter.

The % loss of free amino groups in this study was greater than that reported by Wang and Ismail (2012). Upon Maillard-glycation of WPI with dextran, a loss of only 1.4% free amino groups was observed after 96 h of incubation at 60°C and 0.49 $a_w$, compared to a loss of 26.7% as observed in this study. This difference in % amine blockage between glycated WPI and glycated WPH is likely attributed to differences in reactivity of the folded protein structure of WPI and the unfolded protein structure of WPH. Enzymatic hydrolysis of WPI to produce WPH not only increases the number of terminal amines, but also increases the accessibility of lysine residues. As a result, WPH more readily reacts with dextran and exhibits greater loss of free amino groups compared to WPI.

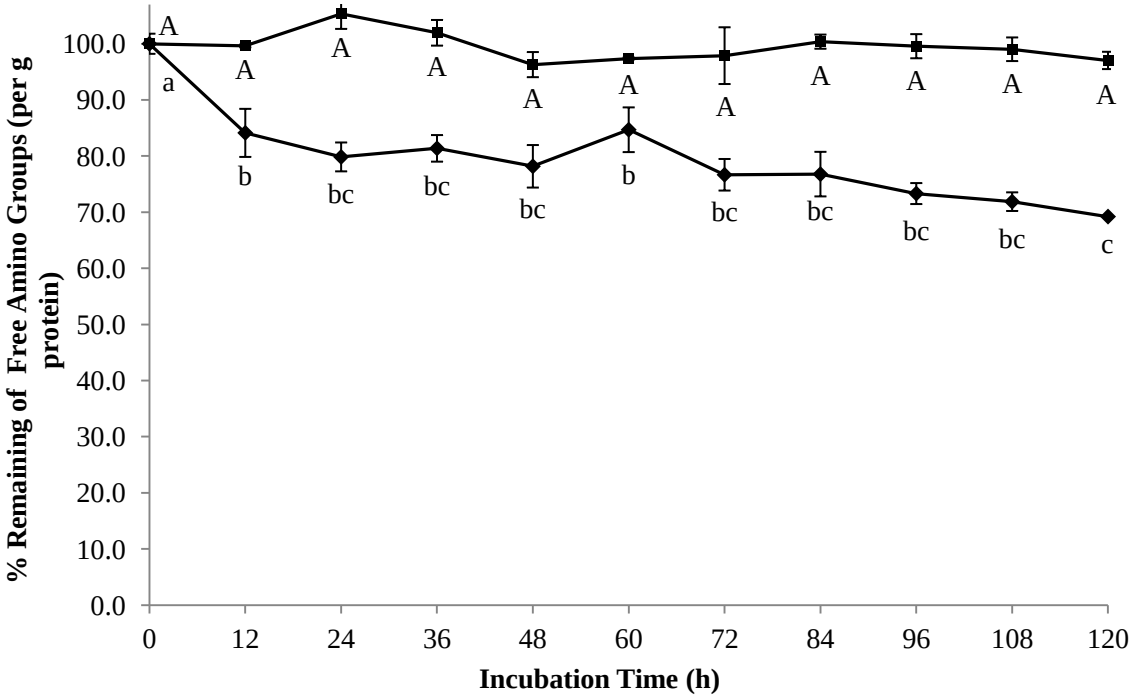


Figure 10. % remaining free amino groups for WPH incubated with dextran at 60°C, 0.49 $a_w$, for 0-120 h, as determined by OPA method. Error bars represent the standard errors (n=3). Different lowercase and uppercase letters indicate significant differences among samples incubated at different time points according to the Tukey-Kraemer multiple means comparison ($P \leq 0.05$).

### 2.4.1.4. SDS-PAGE

SDS-PAGE with Coomassie blue staining was used to visualize the progression of Maillard-glycation based upon the changes in molecular weight of WPH proteins and peptides by the covalent addition of one or more 9-10 kDa dextran per one protein/peptide component. Noticeable reduction in band intensity in the 2-15 kDa range was observed, and a longitudinal smearing in the 15-250 kDa range became more apparent as incubation progressed from 0 to 120 h, indicating a shift to higher molecular weight compounds (Figure 11). The longitudinal smearing is attributed to the

heterogeneous molecular weight distribution of glycated proteins and peptides. Overall, it is apparent that glycation was initiated as early as 12 h, and continued overtime.

Concurrently, SDS-PAGE with glycoprotein staining was used to visualize the formation of glycoproteins due to Maillard-glycation (Figure20, Appendix B). As incubation time increased, smearing in the 20-250 kDa range became more apparent. Non-incubated WPH exhibited little to no smearing in this same range, supporting previous conclusions that Maillard-glycation was initiated within the first 12 h of incubation, and continued to progress overtime. Additionally, the faintness of the smearing indicated that Maillard-glycation was limited to low-levels throughout the incubation time. This is in agreement with observations reported by other researchers, who saw longitudinal smearing after 96 h of incubation of WPI and dextran that was not observed in the control (Wang and Ismail, 2012).

After considering results from UV-Difference spectroscopy, fluorescence spectroscopy, loss of free amino groups, and SDS page, a 48 h incubation time period was selected for further analysis, as this time point resulted in modest Amadori compound formation, as noted by absorbance at 304 nm and % amino group blockage, with minimal progression to intermediate stages, as noted by % fluorescence intensity, and advanced stages of the Maillard reaction, as noted by absorbance at 420 nm.

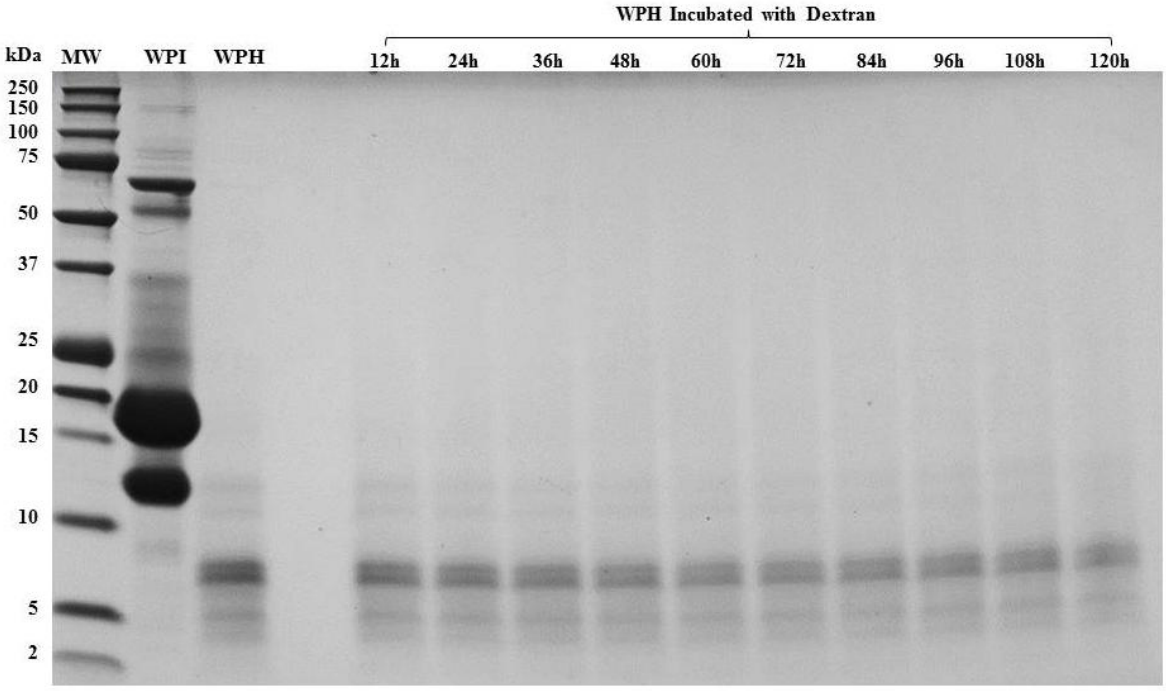


Figure 11. Change in peptide/protein molecular distribution for WPH incubated with dextran at 60°C for 0-120 h at 0.49 $a_w$ as visualized by Coomassie blue stained SDS-PAGE. MW: molecular weight in kDa. Whey protein isolate (WPI) was run as a reference.

### 2.4.2. Separation of PGWPH from Free Dextran

Free, unreacted dextran was removed from the incubated WPH and dextran mixture for several reasons. First, excess free dextran could interfere with further testing. For instance, free dextran may increase turbidity, which would interfere with the spectrophotometric assays, and may also increase viscosity, which would interfere with solubility and thermal stability testing. Additionally, free dextran remains available to take part in the Maillard reaction with the PGWPH, allowing for continued initiation and progression of the reaction beyond initial stages during storage of the ingredient or formulated product/beverage. As a result, undesirable browning, decreased nutritional

quality and changes in functionality could occur. Therefore, separation of free dextran from PGWPH was carried out using ultrafiltration to ensure retention of small molecular weight bioactive peptides, and subsequent HIC, which takes advantage of whey protein's reversible neutralization of charge in high salt concentrations during which hydrophobic regions of the protein adsorb to the hydrophobic column media. When the salt concentration of the mobile phase is reduced, hydrophobic interactions between the protein and column are decreased, the protein desorbs from the column media and elutes from the column.

Previous researchers Wang and Ismail (2012) used 1 M ammonium sulfate solution to allow for the hydrophobic interaction of WPI with the column. In this study, however, the ammonium sulfate concentration was increased from 1 M to 2 M. Upon hydrolysis of WPI, the freed ionizable amine and carboxyl groups increase the charge load, hence using 1 M ammonium sulfate resulted in poor retention on the column and thus poor recovery. Increasing the salt concentration from 1 M to 2 M resulted in enhanced protein-column hydrophobic interactions, increasing PGWPH retention on the column during the free dextran elution phase and, hence, increasing overall protein recovery (Figure 12). Enhanced protein retention was indicated by the reduced peak at retention time 10-20 min and the enlarge peak at retention time 35-60 min (compare figure 12A to 12B).

The amount of 2 M ammonium sulfate needed to elute the free dextran before switching to DDW to elute PGWPH was determined experimentally by using the phenol-sulfuric acid method. When dextran alone was run through the column, it was found that most of it eluted within 21 min of 2 M ammonium sulfate wash (Figure 13). It should be noted that some proteins eluted during the dextran wash, which were hydrolysates that could not effectively be made hydrophobic by the high salt concentration (see peak at retention time 12-20 min, Figure 12B). In order to minimize the loss of proteins and peptides while eluting as much free dextran as possible, a dextran elution time of 22 min was chosen.

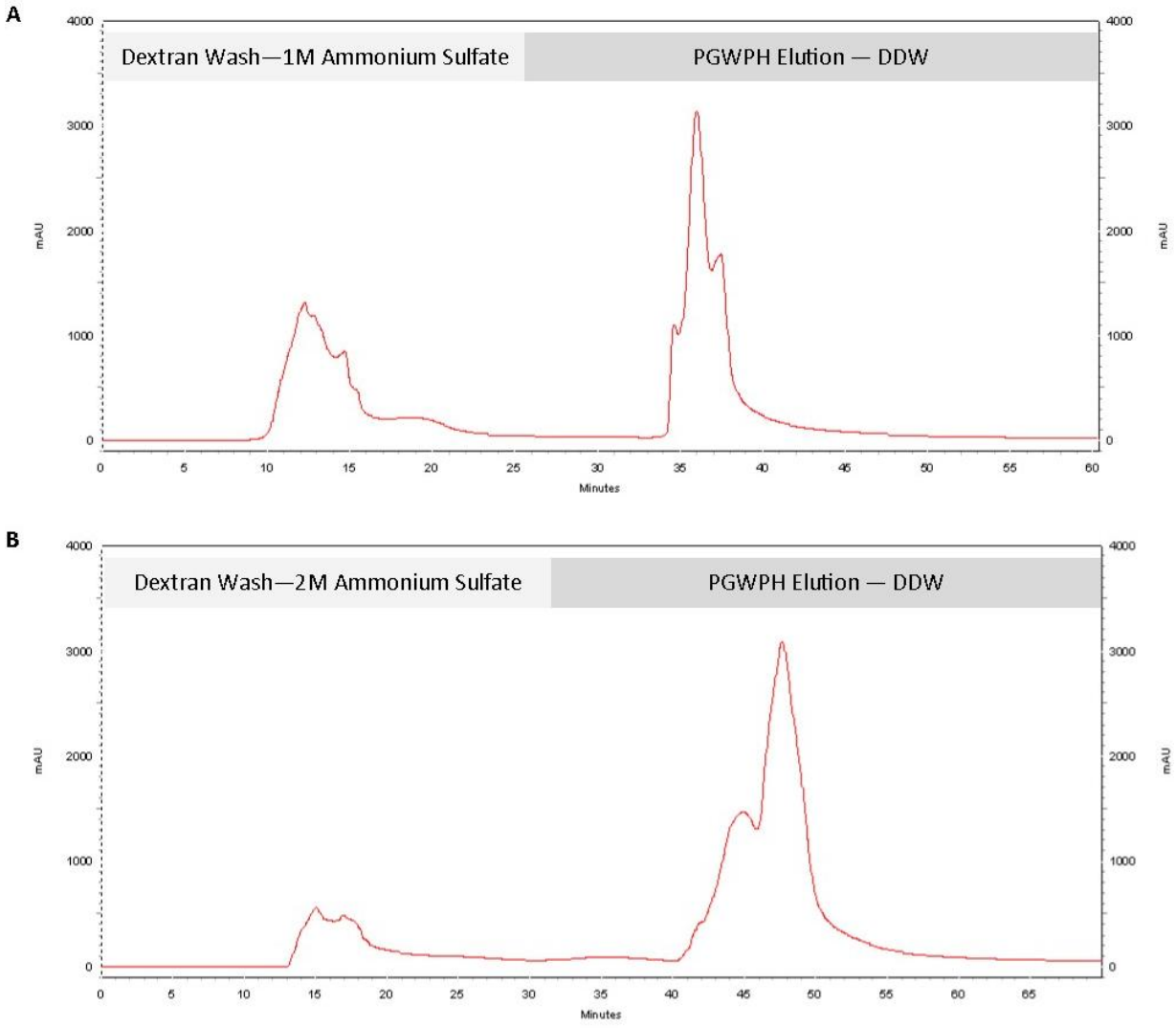


Figure 12. Elution of retentate protein/peptides during separation of free dextran from glycated and non-glycated whey protein and peptides using A) 1M ammonium sulfate and DDW and B) 2M ammonium sulfate DDW (4 mL injection volume, 4.0% protein (w/v)). Chromatograms show absorbance at 220 nm, scaled to 4000 absorbance units.

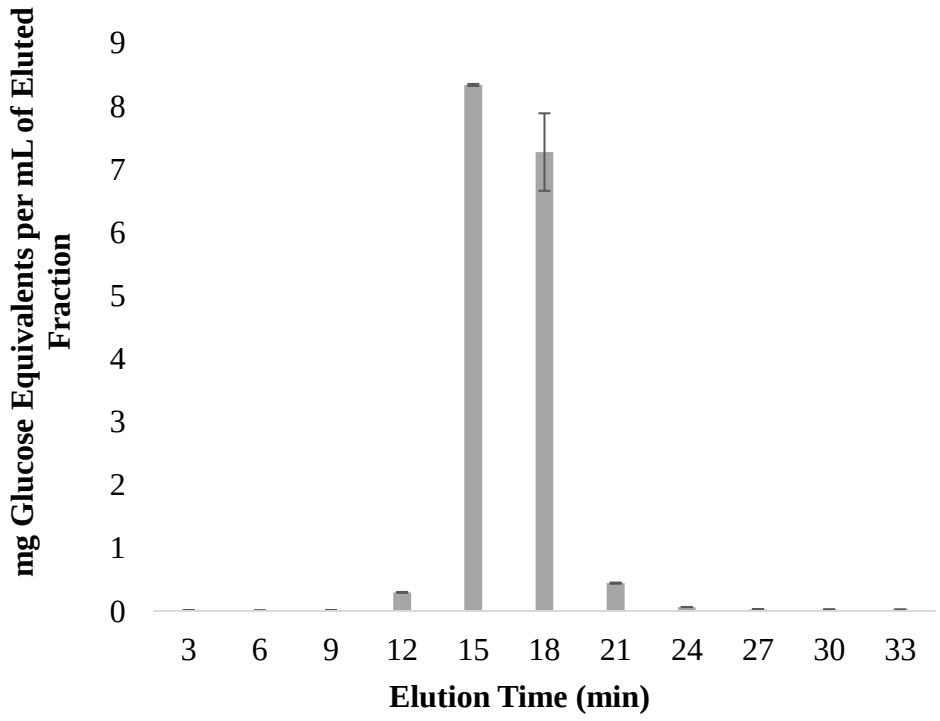


Figure 13. Elution of carbohydrate of a 4:1 mixture of dextran and WPH, showing mg glucose equivalents per mL of fractions collected every 9mL (3min), as determined by the phenol-sulfuric acid method (AOAC Method 988.12). Error bars represent standard errors (n=2).

However, overtime, column fouling occurred, as a moderate amount of protein was strongly interacting with the media and not eluting with the DDW wash. To prevent column fouling, 0.1 N NaOH wash was added after each third injection to elute the strongly bound proteins. This protein fraction was collected, but not combined with the DDW eluted fractions due to the possibility that the protein could be irreversibly denatured, ultimately affecting its functionality. However, the NaOH eluted fraction will be analyzed in the future to test functionality and determine if it may be combined with DDW eluted protein fractions to increase overall protein recovery in the HIC separation procedure.

The yields after the ultrafiltration step, HIC separation and desalting are shown in Table 1. The starting mass of WPH incubated with dextran before the first centrifugation step was 16.5 g, containing 3.3 g of protein. A yield of 96% was obtained after the ultrafiltration step. The small loss in mass is most likely due to transfers and sample fouling on the filtration membranes. After HIC separation and desalting, a total mass of 3.29 g of the original 16.5 g of sample remained. Such a significant loss in mass was expected and attributed to separation and removal of free dextran. In terms of protein, 2.91 g of the original 3.3 g of protein remained, resulting in a protein yield of 88.2%. Protein losses during HIC separation are attributed to proteins that strongly adsorbed to the hydrophobic column media and could only be eluted using 0.1N NaOH.

Table 1. Yields of WPH incubated with dextran after ultrafiltration and after completion of HIC separation and desalting. (Initial Mass: 16.5 g, of which 3.3 g is protein)

| | Total Mass after Ultrafiltration (g) | Total Mass after HIC Separation and Desalting (g) |
|---|---|---|
| Retentate | 15.4 | 2.83 |
| Permeate | 0.463 | 0.463 |
| Total | 15.9 | 3.29[a] (2.91 g protein) |
| % Yield | 96.1% (mass yield) | 19.9% (mass yield)<br>88.2%[b] (protein yield) |

[a] Mass of combined retentate and permeate

[b] 2.910 g of the original 3.3 g of protein remained after separation, resulting in a protein yield of 88.2%.

### 2.4.3. Protein and Carbohydrate Analysis of Purified PGWPH

PGWPH was composed of about 88% protein and 12% carbohydrate (Table 2). The protein content is close to 90%, which is the minimum required to be considered an isolate and is preferred by industry. The carbohydrate content of PGWPH (12.1%) determined by the phenyl sulfuric acid total carbohydrate assay was similar to the carbohydrate content (11.8%) calculated from the difference in total mass and protein mass, as determined by DUMAS. Given that the free dextran was eluted prior to the protein/peptide collection, the measured total carbohydrate in the sample is most likely attributed to dextran conjugation with the protein/peptides. The data, therefore, confirms that partial glycation did occur under the limited and controlled Maillard reaction conditions used.

Table 2. Protein and Carbohydrate Composition of PGWPH and WPH

| | % Protein (dry basis) ± SE[a] | % Carbohydrate ± SE[a] |
|---|---|---|
| PGWPH | 88.4 ± 0.2 | 12.12 ± 0.03 |
| WPH[b] | 94.9 ± 0.8 | < 1.0 |

[a] Standard Error [b] From manufacturer's specifications

### 2.4.4. Changes in Protein Solubility

At a protein concentration of 5%, WPH exhibited excellent solubility at pH 3.4 and 5.5 (Table 3). However, its solubility dropped to 46% near its pI (pH 4.5). After heating for 30 min at 80°C, WPH maintained excellent solubility at pH 3.4, but had significantly ($P \leq 0.05$) lower solubility at pH 4.5 and 5.5. Comparatively, under the same conditions, Wang and Ismail (2012) observed that WPI post similar heat treatment had solubility of 3.9% and 7.3% at pH 4.5 and 5.5, respectively. The higher solubility of

WPH compared to WPI around the isoelectric point post thermal treatment could be attributed to reduced molecular weight and increased hydrophilicity resulting from the increase in free carboxyl and amine groups upon hydrolysis. However, hydrophobic interactions and disulfide linkages may still take place upon heating, leading to aggregation and, hence, the observed decrease in solubility compared to unheated WPH.

PGWPH exhibited excellent solubility at all pHs tested. Upon heating, PGWPH maintained its high (≥ 90%) solubility, even at pH 4.5. In contrast to what was observed for WPH, the maintained solubility, particularly around the pI, is due to several contributing factors, including increased net negative charge that results in increased electrostatic repulsion, increased overall hydrophilicity that results in enhanced protein-water interaction, and delayed onset of denaturation (Wang and Ismail, 2012). Furthermore, the bulkiness of dextran increased steric hindrance, further decreasing protein-protein interaction.

Table 3. Percent solubility of whey protein hydrolysate (WPH) and partially glycated whey protein hydrolysate (PGWPH) at 5% protein concentration and various pH, non-heated and heated at 80°C for 30 min.

| | | **% Protein (w/v)** | |
|---|---|---|---|
| **pH** | **Heat Treatment** | **WPH** | **PGWPH** |
| 3.4 | Non-heated | 102.3 A | 95.7 A |
| | 80°C | 106.0 A | 90.2 A |
| 4.5 | Non-heated | 48.3 C | 103.7 A |
| | 80°C | 46.8 C | 91.0 A |
| 5.5 | Non-heated | 98.4 A | 99.1 A |
| | 80°C | 60.9 B | 92.7 A |

Means in each column with different uppercase letters indicate significant differences in solubility across different pHs according to the Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

The enhanced solubility of PGWPH at pH close to the pI under heated and non-heated conditions was in agreement with previous research on non-hydrolyzed whey protein conjugates (Akhtar and Dickinson, 2007; Zhu et al., 2010; Wang and Ismail, 2012). However, the observations in this study confirmed that the enhanced solubility of intact whey proteins due to glycation is similarly exhibited upon glycation of whey protein hydrolysate. The results also indicated that partial and limited Maillard-induced glycation of whey protein hydrolysate obtained under the mild conditions employed, is sufficient to cause significant enhancement of WPH solubility in heated solutions over a wide range of pH and at protein concentration > 4.2%.

These results make the use of PGWPH in acidified whey protein beverages very promising. The use of PGWPH, in place of WPH or WPI, will increase thermal stability of the beverage, allowing for protein concentrations great than 4.2% and hence the ability to meet the requirements for a "high protein" claim. Furthermore, since PGWPH exhibited enhanced solubility at pH 4.5 compared to WPH at the same conditions, acidic whey protein beverages could be formulated at or near pH 4.5, rather than lower pHs (typically pH 3.4) that are currently used in industry. Formulating at a higher pH would require less addition of acid to the beverage, thus reducing beverage sourness and astringency as well as increasing overall consumer acceptance of acidified whey protein beverages.

### 2.4.5. Surface Hydrophobicity and Total Sulfhydryl Groups (SH)

Changes in surface hydrophobicity were used to assess thermal denaturation of WPI, WPH and PGWPH, and to assess potential for hydrophobic interactions, which contribute to aggregation and precipitation. The initial surface hydrophobicity index of WPI prior to heating was significantly ($P \leq 0.05$) lower than that of WPH (Figure 14) due to the fact that most hydrophobic groups are buried within the interior of the globular protein's intact structure. However, as heating time increased, the surface hydrophobicity index of WPI gradually increased and exceeded that of WPH. The increase in

hydrophobicity of WPI reflected the exposure of hydrophobic regions originally buried inside the protein globular structure. After 20 min of heating, the surface hydrophobicity of WPI peaked and began gradually decreasing, most likely due to the formation of intermolecular hydrophobic interactions, reducing the number of hydrophobic groups exposed on the surface of the proteins (Sava et al., 2005).

On the other hand, the increase in surface hydrophobicity of WPH upon heating was much lower than that of WPI, and did not exhibit such a large increase in surface hydrophobicity during heating as did WPI. Since WPH has already undergone some degree of denaturation due to enzymatic hydrolysis, the protein structure was more resistant to heat denaturation, and intermolecular hydrophobic interactions may have already occurred during industrial production of WPH. While there was a significant increase post heating at 75°C for 10 min for WPH, no significant increase was noticed with increased heating time. After 60 min of heating, a decrease in surface hydrophobicity of WPH was not observed. This is likely attributed to the freed ionizable amine and carboxyl groups, which increase the charge load and increase electrostatic repulsion. As a result, protein-protein interaction is reduced, and hydrophobic interactions are reduced.

The overall surface hydrophobicity of PGWPH was much lower than that of WPI and WPH, and was not greatly affected by heat. A significant increase, yet limited, in exposure of new hydrophobic groups was observed after 30 min of heating, 20 min later than what was observed for WPH. This is likely due to various physical and chemical changes that occurred with glycation resulting in increased resistance to thermal denaturation, as earlier discussed. The difference in the surface hydrophobicity index between WPH and PGWPH is likely due to the blockage of hydrophobic regions upon glycation, as observed by previous researchers (Nacka et al., 1998; Mu et al., 2006; Wang and Ismail, 2012), and more notably, due to the loss of the strongly hydrophobic proteins/peptides during HIC separation that would not elute in DDW. After 60 min of heating, the surface hydrophobicity of PGWPH did not decrease. Similar to WPH, this is likely attributed to the freed ionizable amine and carboxyl groups, which increase the

charge load and increase electrostatic repulsion, as discussed earlier. In the future, extended the heating time and increasing the heating temperature would be recommended to determine if a decrease in hydrophobic groups would occur at these new parameters.

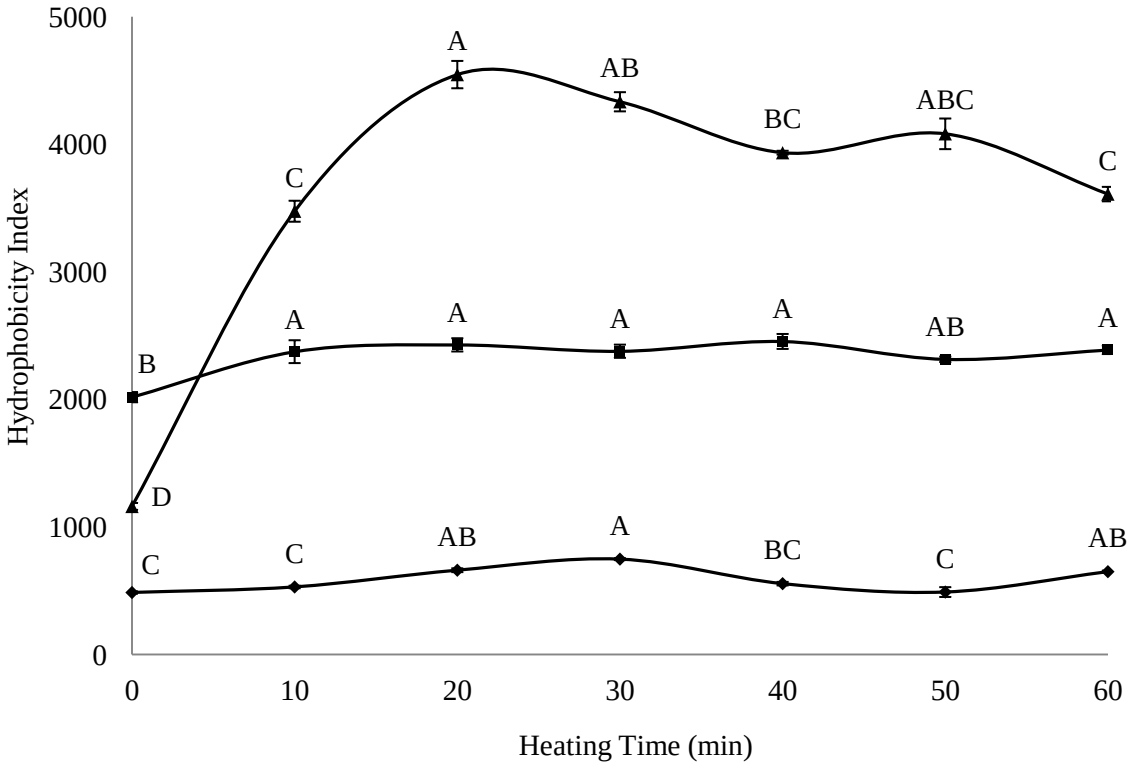


Figure 14. Surface hydrophobicity index of 5% whey protein isolate (WPI) (▲), whey protein hydrolysate (WPH) (■), and partially glycated whey protein hydrolysate (PGWPH) (◆), solutions heated at 75°C for 0-60 min. Error bars represent standard error; n=3. Different uppercase letters above the bars indicate significant differences across different time points within each sample set according to the Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

Similarly, changes in surface sulfhydryl groups (SH) were also used as an indicator of thermal denaturation for WPH and PGWPH and potential for formation of disulfide linkages. For WPI, Wang and Ismail (2012) observed that upon heating the

amount of surface SH groups significantly ($P \leq 0.05$) increased from 92 μM cysteine, reaching a maximum of 155 μM cysteine after 50 min of heating, and then decreased as heating time increased. The increase in SH groups upon heating was due to the unfolding of the protein and exposure of free SH groups. As heating continued, a decrease in free SH groups was attributed to the formation of disulfide linkages. In this study, the exposure of free SH groups of WPH gradually increased upon heating, but did not decrease (Figure 15). It also had a lower initial cysteine level than that observed for WPI by Wang and Ismail (2012). PGWPH had a slight decrease in surface sulfhydryl groups upon heating, followed by relatively no change upon further heating until 60 min when a slight increase was observed. The lack of gradually exposure of free SH groups of PGWPH compared to WPH is again likely due to resistance to thermal unfolding upon glycation (Wang and Ismail, 2012; Wang et al., 2013). As observed for surface hydrophobicity index, the difference in the surface sulfhydryl groups between WPH and PGWPH could be attributed to the loss of some proteins/peptides during HIC separation.

The observed surface hydrophobicity and free SH data suggest that glycation enhances thermal stability of WPH, as PGWPH was more resistant to heat-induced unfolding. The minimal exposure of sulfhydryl groups and the hydrophobic residues upon heating of PGWPH will help reduce protein aggregation and contribute to thermal stability of whey protein hydrolysates in beverage applications at protein concentrations greater than 4.2%.

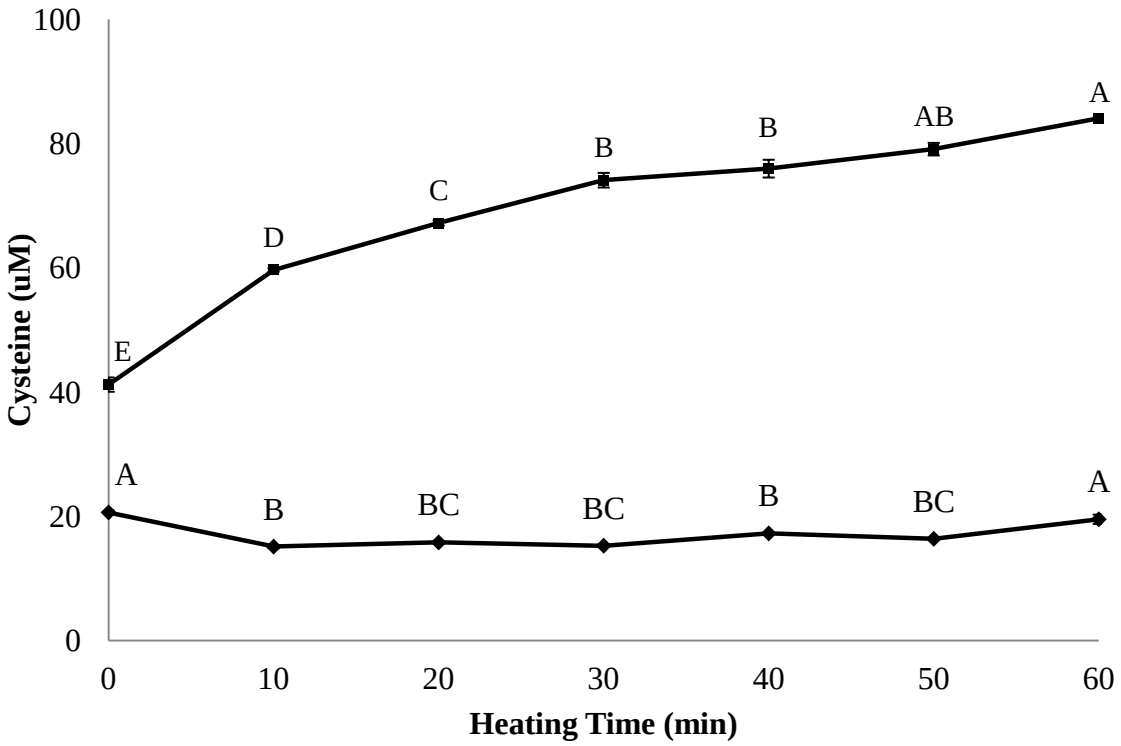


Figure 15. Surface sulfhydryl groups of 5% whey protein hydrolysate (WPH) (■) and partially glycated whey protein hydrolysate (PGWPH) (♦) solutions heated at 75°C for 0-60 min. Error bar represents standard error; n=3. Different uppercase letters above the bars indicate significant differences across different time points within each sample set, according to the Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

### 2.4.6. Heat-Induced Polymerization

A slight increase in band intensities near 250 kDa was noted as heating time increased from 0 to 60 min for the WPH samples (Figure 16A). High molecular weight bands were observed in the 0 min samples, indicating that the original WPH had some disulfide linkages due to thermal treatment during manufacturing. However, no noticeable change in band intensities was noted as heating time increased from 0 to 60 min for the PGWPH samples. The high molecular weight bands disappeared when samples were run under reducing conditions (Figure 16B), confirming the formation of disulfide linkages upon heating. Although disulfide polymerization upon heating of

PGWPH did occur, it was less pronounced than that observed for heated WPH samples, especially with prolonged heating. This observation can be partially explained by the fact that PGWPH resisted further heat-induced unfolding and complete denaturation compared to WPH. Thus, less hydrophobic regions and free sulfhydryl groups were exposed over the heating time, as discussed earlier, reducing the chances for hydrophobic interactions and disulfide linkages to occur between the protein molecules. Additionally, PGWPH would exhibit more electrostatic repulsion due to the increased net negative charge in addition to steric hindrance, thus further limiting protein-protein interactions and possible changes in disulfide linkages.

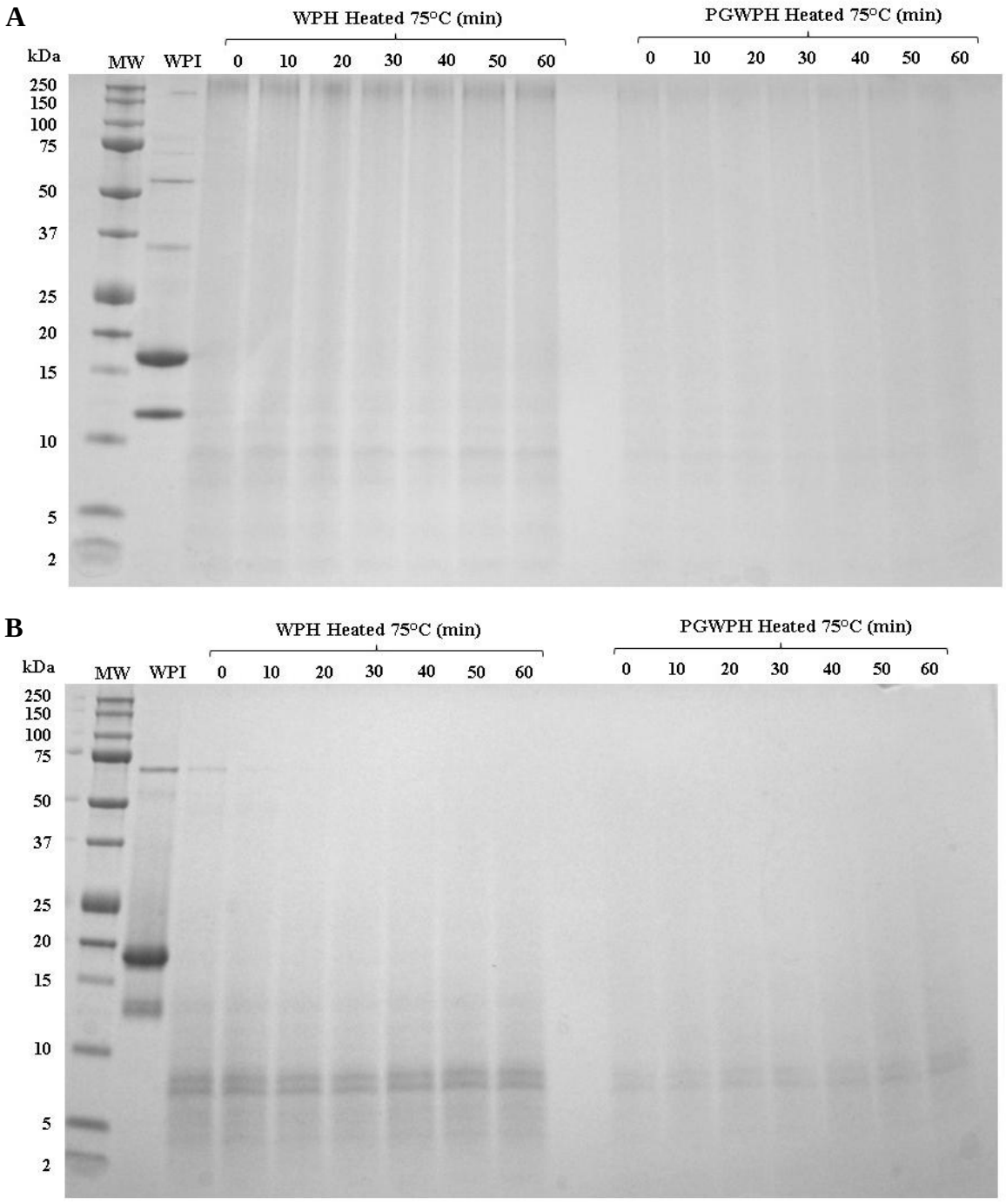


Figure 16. Change in peptide/protein molecular distribution as visualized by Coomassie blue stained SDS-PAGE for WPH and PGWPH heated at 75°C for 0-60 min and run under A) non-reducing and B) reducing conditions. MW: molecular weight in kDa. Whey protein isolate (WPI) was run as a reference.

## 2.5. Conclusions

The Maillard-induced glycation conditions utilized in this study optimized Amadori compound formation, while limiting the propagation of Maillard reaction to intermediate and advanced stages. WPH incubated with dextran for 48h was chosen for further study due to its moderate formation of Amadori compounds, low browning and fluorescent compound formation, and only moderate reduction in free amino groups. Removal of free dextran from glycated and non-glycated protein and peptides was carried out using ultrafiltration and hydrophobic interaction chromatography (HIC) with success. The final composition of PGWPH was approximately 88% protein and 12% carbohydrate. Purified glycated whey protein hydrolysate displayed enhanced solubility and thermal stability at 5% protein concentration under acidic pH conditions. The enhanced solubility and thermal stability of PGWPH, even when the pH was close to the pI of the whey protein, was attributed to the resistance to denaturation and structural modifications. Overall, this work has shown for the first time that partial Maillard glycation can be induced and controlled to low-levels in whey protein hydrolysate, producing a value-added product with potentially enhanced functionality and stability. Acidified whey protein beverages could be formulated with PGWPH in place of WPH or WPI may have a longer shelf life, a more acceptable flavor, and protein content greater than 4.2%, allowing for a "high protein" beverage claim to be made. In turn, the utilization of biologically active WPH in acidified protein beverages would greatly increase, and consumer demands for a functional, high protein beverage could be fulfilled.

# 3. Effect of Maillard-Induced Glycation on the Nutritional Quality, Bioactivity and Allergenicity of Whey Protein Hydrolysate

## 3.1. Overview

This study examined the changes in nutritional quality, bioactivity and allergenicity of whey protein hydrolysate (WPH) subjected to controlled and limited Maillard-induced glycation using dextran. WPH was subjected to Maillard-induced glycation under controlled conditions to produce partially glycated WPH (PGWPH). Ultrafiltration and hydrophobic interaction chromatography (HIC) were used to separate the non-glycated and glycated whey protein/peptides from the unreacted, free dextran. Nutritional quality, bioactivity, and immunoreactivity of WPH were assessed before and after glycation. Nutritional quality was assessed using a furosine assay to determine lysine blockage, and a sequential pepsin-trypsin *in-vitro* digestion assay to determine digestibility. Bioactivity, namely antihypertensive activity, was determined using an *in-vitro* angiotensin converting enzyme (ACE) inhibition assay. Changes in immunoreactivity were assessed following an indirect enzyme-linked immunosorbent assay (ELISA) using sera from milk sensitive donors. Nutritional quality and bioactivity of WPH was minimally impacted upon partial glycation, as lysine blockage was only 2%, and digestibility (58.7%) and antihypertensive activity of PGWPH ($IC_{50}$ =0.249) were similar to that of WPH. However, while WPH had reduced immunoreactivity (40-75% reduction) compared to WPI, glycation did not further reduce its immunoreactivity. These results indicate that partial glycation of WPH does not limit the application of WPH as a nutritional and bioactive food ingredient, but under the current glycation conditions, does not have the potential to further reduce allergenicity of WPH for use in hypoallergenic food applications. Further optimization of glycation conditions is needed to reduce the allergenicity of WPH.

## 3.2. Introduction

With increasing consumer interest in functional, better for you foods, whey protein ingredients are becoming very popular due to their nutritive and physiological health benefits. However, one of the major hurdles for expanded use of whey protein in food applications is its allergenicity. In the United States, food allergy prevalence is on the rise, increasing by 50% since 1997, and projected to continue increasing (Branum and Lukacs, 2008; Jackson et al., 2013). In particular, cow's milk allergy appears to be among the more prevalent food allergies in infants and children, with a rate of 2.5% (Sicherer and Sampson, 2010). Over the years, the increase in observed incidences of milk allergies, and more specifically whey protein allergies, is thought to be due to the increased popularity and use of milk products by the population and the introduction of whey protein-based products to many different food categories. The increased exposure to whey protein has led to increasing rates of sensitization, accidental ingestion, anaphylaxis, and even death in whey-allergic individuals (Liu et al., 2010).

Allergic reactions are caused by presence of specific sequences of amino acids, called epitopes, in the native protein. These epitopes are recognized by the immune system immunoglobulin (IgE) antibodies, which then elicit an immune response. Therefore, protein modification techniques are needed to reduce or eliminate allergenicity of whey protein ingredients by destroying epitopes or preventing IgE binding to the epitope sites. Several different protein modification techniques have been studied, including enzymatic hydrolysis and Maillard-induced glycation.

Enzymatic hydrolysis can be used to cleave and disrupt linear epitopes and collapse conformational epitopes, resulting in reduced immunoreactivity. Hydrolysis of whey proteins not only reduces allergenicity, but also contributes physiological benefits attributed to bioactive peptides. Bioactive peptides are short amino acid sequences that upon release from the parent protein by hydrolysis may contribute several physiological properties. Reported biological activities of whey peptides include gastrointestinal functions, anticarcinogenicity, antimicrobial activity, growth promotional activity,

immunoreactivity, and anti-hypertensive activity (Meisel et al., 1989; Gonzàlez-Tello et al., 1994; FitzGerald and Meisel, 1999; Walzem, 1999; Shah, 2000; Pihlanto-Leppala, 2001; Ha and Zemel, 2003; Smithers, 2008).

Antihypertensive activity is one of the most researched properties of whey derived bioactive peptides, as hypertension is a prominent health issue in the U.S. Roughly 1 in 3 adults in the U.S. have hypertension, and of those, less than half have it under control (CDC, 2012). Hypertension can damage blood vessels and the heart, increasing risk for coronary heart disease and stroke, which are two of the leading causes of death for Americans (Kochanek et al., 2011). Overall, treatment of hypertension costs Americans over $47.5 billion each year (Heidenreich et al., 2011). This makes bioactive peptides with antihypertensive properties all the more important to research.

Furthermore, enzymatic hydrolysis increases the nutritional properties of the whey protein. WPH is considered a superior alternative to WPC or WPI because the peptides are easier to digest (Nnanna and Wu, 2007). Studies have also shown that the amino acids and peptides in WPH are more readily absorbed into the bloodstream than other proteins and amino acid solutions, enhancing the recovery process of athletes (Boza et al., 2000; Buckley et al., 2010).

However, problems with enzymatic hydrolysis do exist, as it is possible that protein unfolding due to hydrolysis can lead to increased accessibility of hidden epitopes within the protein structures (L'Hocine and Boye, 2007). These newly accessible epitopes are then available for IgE binding. However, extensive enzymatic hydrolysis (degree of hydrolysis (DH) > 25%) is very effective at reducing allergenicity of whey proteins (Nielsen, 2009; Bu et al., 2013). Unfortunately, such extensively hydrolyzed product rarely has any food applications other than infant formulas due to detrimental effects on functionality and flavor. The loss of protein structure exposes hydrophobic groups and free sulfhydryl groups, increasing hydrophobic and disulfide interactions between whey proteins and their peptides, leading to polymerization, aggregation and precipitation. Furthermore, hydrolysis will result in the release of low molecular weight,

hydrophobic bitter peptides, leading to decreased consumer acceptance. In contrast, limited hydrolysis (< 8%) releases some beneficial bioactive peptides while maintaining functionality, and may reduce allergenicity of whey protein. However, limited hydrolysis alone is not sufficient to eliminate allergenicity.

Maillard-induced glycation is another protein modification technique that has been studied for reducing protein allergenicity (L'Hocine and Boye, 2007). The potential of the Maillard reaction to reduce allergenicity of a protein is promising, as the allergenicity of several protein sources such as soy (Wilson et al., 2005; van de Lagemaat et al., 2007), buckwheat (Nakamura et al., 2008), egg (Ma et al., 2013), hazelnuts (Iwan et al., 2011), peanuts (Gruber et al., 2005) and cherries (Gruber et al., 2004) has been largely reduced by glycation with a variety of carbohydrates sources. Moderate degrees of glycation resulted in only a small reductions in allergenicity, whereas higher degrees of glycation have resulted in up to 99% reduction (Kobayashi et al., 2001; Bu et al., 2010; Li et al., 2011). The reduction in allergenicity by glycation is due to blockage of the epitope sites from sugar or polysaccharide binding, or the shielding effect of the sugar or polysaccharide that prevents access of IgE to the epitopes (Usui et al., 2004; Taheri-Kafrani et al., 2009; Bu et al., 2013). However, with successful reduction in allergenicity, a high degree of glycation can be detrimental to the overall quality of the protein for use in food applications. Excessive glycation results in nutritional quality loss due to extent of lysine blockage. Additionally, propagation of the Maillard reaction to advanced stages not only leads to the excessive formation of melanoidins, but also leads to polymerization, which results in aggregation and reduced digestibility (Erbersdobler et al., 1981; Hidalgo and Zamora, 2000; Panza et al., 2010; Zhou et al., 2013). Additionally, excessive glycation could potentially negatively affect bioactivity. It is, therefore, important to control and limit the rate of glycation in order to produce a partially glycated whey protein product with minimal nutritional loss and maintain bioactivity.

While enzymatic hydrolysis can release bioactive peptides and greatly reduce allergenicity of whey protein, the functionality and flavor quality of the protein may be sacrificed. Similarly, extensive Maillard-induced glycation has been shown to

significantly reduce allergenicity of proteins, however overall quality and bioactivity may be sacrificed. It is hypothesized that combining the two mechanisms, limited enzymatic hydrolysis and limited Maillard glycation under controlled and mild conditions, may have a synergistic effect on reducing allergenicity of whey protein while maintaining nutritional quality and bioactivity. Therefore, the objective of this work was to produce a partially-glycated whey protein hydrolysate following controlled and limited Maillard-induced glycation and determine its resulting nutritional quality, bioactivity (anti-hypertensive activity) and allergenicity.

## 3.3. Materials and Methods

### 3.3.1. Materials

All materials are the same as listed in Section 2.3.1., with some additions. Furosine standard was purchased from PolyPeptide Group laboratories (San Diego, CA, USA). Sep-Pak C18 filter cartridges (WAT023501) were purchased from the Waters Corporation (Milford, MA, USA). Kimax vial (13 x 100 mm) with PTFE faced septa caps (45066A) were purchased from Kimble Chase (Vineland, NJ, USA). Angiotensin converting enzyme from rabbit lung (2.0 units/mg), N-[3-(2-Furyl)acryloyl]-Phe-Gly-Gly (FAPGG), pepsin from porcine gastric mucosa (EC 3.4.23.1 , 3,200-4,500 units $mg^{-1}$, P6887), trypsin type 1X-S from porcine pancreas (EC 3.4.21.4, 10,000 BAEE units $mg^{-1}$, T8003) were purchased from Sigma Aldrich (St. Louis, MO, USA). Biotinylated goat anti-human IgE (16-10-04) was purchased from Kirkland and Perry Laboratories (Gaithersburg, Maryland). Polystyrene Costar 96-well microplates (3370) were purchased from Corning (Tewksbury, MA). Streptavidin alkaline phosphatase (s2890) and p-nitrophenol phosphate (N7653) were purchased from Sigma-Aldrich. All other chemical grade reagents were purchased from Fisher Scientific (Waltham, MA) or Sigma-Aldrich.

### 3.3.2. Preparation of PGWPH

PGWPH was prepared in the same manner as outlined in section 2.3.2 and purified in the same manner as outlined in section 2.3.4.

### 3.3.3. Determination of Lysine Blockage

Lysine blockage of the 48 h incubated PGWPH sample before HIC separation was determined by measuring 2-furoyl-methyl-Lys (furosine), the Amadori compound formed upon the reaction of a lysine residue with a reducing sugar (Guerra-Hernandez et al., 2002), following the hydrolysis procedure outlined by Jiménez-Castaño et al. (2007) and HPLC analysis reported by Krause et al. (2003), with some modifications. PGWPH and WPH were analyzed in triplicate. For each, 3.75 mL of 7.95 N HCl was added to 0.02 g protein in a 13 x 100 mm vial. The vials were flushed with nitrogen, sealed, and heated at 43.3°C in an Equatherm TempBlok® heating block (Lab-Line Instruments, Thrippunithura, India) for 24 h. After digestion, samples were cooled to room temperature, supernatants were removed and centrifuged at 10,000 x g for 1 min to settle any debris from the acid digestion. Supernatants were then filtered through a 0.45 µm PVDF low binding filter. Each filtrate (0.5 mL) was applied to a previously activated Sep-Pack $C_{18}$ light cartridge at a rate of 0.5 mL/min. Furosine was eluted with 2 mL of 3 N HCl and neutralized with 0.33 mL of 18 N NaOH. To quantify furosine content, an HPLC system (Shimadzu Scientific Instruments, Columbia, MD, USA) equipped with SIL-10AF auto injector, SPD-M20A photo diode array detector and a CTO-20A column oven was used. A YMC pack ODS AM-12S05-2546WT C-18 reverse phase column (250 mm x 4.6 mm, 5 µm) and a guard column (20 mm x 4 mm) of the same material were used. Column temperature and flow rate were maintained at 35°C and 1.2 mL/min, respectively. Solvent A was 5 mM sodium heptanesulphonate and 0.2% formic acid in DDW, and solvent B was 80% acetonitrile (ACN) containing 5mM sodium heptanesulphonate and 0.2% acetic acid. Following the injection of 20 µL of a sample,

elution was performed at 15% ACN (81.3% Solvent A: 18.7% Solvent B). A standard curve was prepared using known furosine standard concentrations (from 0.5 to 20 ppm). UV detection was measured at 280 nm. Furosine was quantified based upon its peak area at the retention time of approximately 8.3 min and transferred into amount of blocked lysine per mole of protein using a series of calculations and conversions. A sample calculation can be found in Appendix C.

### 3.3.4. Determination of *In-vitro* Digestibility

Digestibility of WPH and the 48 h incubated PGWPH sample post HIC separation was determined, in duplicate, following the sequential *in-vitro* digestion by pepsin and trypsin as outlined by Tang et al., (2006), with modifications. Solutions (10 mL of 1% protein, w/v) of PGWPH and WPH were prepared in 0.1 N HCl at a pH of 1.5 and warmed up to 37°C in a water bath with gentle stirring. After 10 min of incubation, pepsin (100 µL of 3 mg/mL) was added to the samples and incubation continued at 37°C for 2 h. The pH was then adjusted to 7.0 using 1.0 N NaOH, to terminate pepsin activity, before trypsin (100 µL of 9 mg/mL) was added to each of the samples and incubated for another 2 h at 37°C. The pH was periodically checked and adjusted during incubation to maintain pH 7.0. After the digestion, the samples were removed from the water bath and filtered through 0.45 µm syringe filters and immediately frozen to -20°C until analysis.

The peptide profiles of the samples were analyzed following a method outlined by Chevalier et al. (2001b), with modifications. A Shimadzu HPLC system and column, as described in section 3.3.3, was used. A linear binary gradient was employed using HPLC grade water (solvent A) and acetonitrile (solvent B), with both containing 0.11% (v/v) trifluoroacetic acid. After injecting 50 µL of sample, solvent B was linearly increased from 16% to 40% in 20 min, kept constant for 10 min, increased to 80% for 10 min, decreased to 16% in 10 min, then followed by column equilibration steps. Throughout the run, the column temperature was maintained at 35°C and the flow rate was kept constant

at 1.2 mL/min. The eluted peptides and proteins were monitored at 214 and 280 nm, respectively. The percent digestibility was determined based on the differences in select peak areas obtained at 214 nm. To calculate % digestibility, **Equation 4** was used.

**Equation 4:**

$$\%\ \text{Digestibility} = \frac{\text{peak area}_{\ \text{non-digested}} - \text{peak area}_{\ \text{digested}}}{\text{peak area}_{\ \text{non-digested}}} * 100\%$$

### 3.3.5. Measurement of Angiotensin-Converting Enzyme (ACE) Inhibitory Activity

The ACE inhibitory activities of WPH and PGWPH pre and post HIC separation were determined in triplicate, following the assay outlined by Otte et al. (2007) and Shalaby et al, (Shalaby et al., 2006) and modified by Margatan et al. (2013) using furanacryloyl-L-phenylalanylglycylglycine (FA-PGG) as the ACE substrate and sodium borate buffer (0.1 M sodium tetraborate with 0.3 M sodium chloride, pH 8.3 at 37°C). WPH and PGWPH samples (10 mg/mL) were prepared fresh before each assay in DDW, centrifuged at 15,682 x g for 10 min, and filtered using 0.45 μm syringe filters. The filtered solutions were further diluted to concentrations of 20, 17.5, 15, 12.5, 10, 7.5, 5, 2.5, 1.75 and 1 mg solids/mL. Protein concentration of the samples was determined using the BCA assay kit, following manufacturer instructions. WPH or PGWPH samples, ACE (0.288 units/mL of DDW), and FAPGG (0.88 mM prepared in 0.1M sodium borate buffer) were warmed to 37ºC and added to a 96-well plate (as outlined in Table 4). Absorbance at 340 nm was measured every 30 sec for 30 min using a microplate reader (BioTek Synergy HT, BioTek Instruments, Winooski, VT, USA) at 37°C. Control and sample blank absorbances (Table 3) were subtracted from those of respective control and samples to obtained corrected absorbances.

The ACE inhibitory activity of WPH and PGWPH was expressed as the slope of the decrease in absorbance at 340 nm during incubation at 37°C. The $IC_{50}$ values (protein

concentrations of WPH or PGWPH that inhibit ACE by 50% in comparison to the control) were determined by plotting % ACE inhibition by protein concentration. The % ACE inhibition by sample solutions was calculated using **Equation 5** and **Equation 6**. The $IC_{50}$ value was deduced from the plot of % ACE inhibition activity vs. the protein concentration of WPH or PGWPH using the equation of the ACE inhibition line obtained using linear regression (**Equation 7**). A sample calculation can be found in appendix D.

**Equation 5:**

$$\text{Conc. of protein in the well } = \frac{\text{protein concentration from BCA (mg/mL)} * 10\ \mu\text{L}}{170\ \mu\text{L}}$$

Where:

Protein concentration from BCA (mg/mL) is the protein concentration determined by BCA for each corresponding inhibitor solution added in the ACE assay

**Equation 6:**

$$\%\ \text{ACE Inhibition} = \frac{(\text{slope}_{\text{control}} - \text{slope}_{\text{inhibitor}})}{\text{slope}_{\text{control}}} * 100\%$$

**Equation 7:**

$$y = mx + b$$

$$IC_{50}\text{value} = x = \frac{(y - b)}{m}$$

Where:

x = protein concentration (mg/mL) of WPH or PGWPH in the well needed for 50% inhibition

m = slope of the ACE inhibition line

b = y-intercept of the ACE inhibition line

y = % ACE inhibition; y = 50 to determine concentration of WPH or PGWPH needed to result in 50% inhibition

Table 4. Reaction Mixture for ACE Activity Assay

| | **Na-borate buffer[a] (μL)** | **FA-PGG[b] (μL)** | **Sample[c] (μL)** | **ACE[d] (μL)** |
|---|---|---|---|---|
| Control blank | 170 | — | — | — |
| Control | 10 | 150 | — | 10 |
| Sample blank | 160 | — | 10 | — |
| Sample | — | 150 | 10 | 10 |

[a] Sodium tetraborate buffer: 0.1 M borate, 0.3 M chlorine ion, pH 8.3

[b] 0.88 mM FA-PGG in Na-borate buffer

[c] Diluted concentrations of WPH or PGWPH

[d] 0.288 units/mL ACE in DDW

### 3.3.6. Patient Recruitment for Determining Allergenicity of PGWPH

All sera and clinical laboratory services for this work were provided by the Allergy Clinic at the Mayo Clinic (Rochester, Minnesota, USA) and Dr. Scott Sicherer from Allergy and Immunology Clinic at Mount Sinai Hospital (New York, New York, USA). Patients with a reported allergy to milk protein were initially screened by reviewing skin prick and/or radioallergoabsorbent test (RAST) results found in their

medical charts. Once a convincing history of an allergy to milk protein was confirmed, research participants were subjected to an initial blood draw. Up to 100 mL of blood was drawn at once or on multiple visits depending on the body weight. Sera was separated from whole blood by centrifugation, aliquoted, and stored at -40°C until later use in Enzyme-Linked Immunosorbent Assay (ELISA) to determine the immunoreactivity of the modified and non-modified whey protein samples. Sera with enough sample to spare were then sent to Quest Diagnostics (Woodale, IL, USA) for testing to determine the whey specific IgE, IgE total, and IgG total, otherwise milk specific IgE levels were tested by Dr. Scott Sicherer. A total of 6 participants with milk protein specific IgE levels of 3.5 kU/L or greater were chosen for participation in this project (Table 5). A specific IgE level of 3.5 kU/L or greater is considered a high level according to the manufacturer of the ImmunoCap assay that was used by Quest to determine whey specific IgE concentrations.

Table 5. Participants' Sera Specific IgE, IgE total, and IgG total levels

| **Participant** | **IgE Milk Specific (kU/L)** | **IgE Whey Specific (kU/L)** | **IgE Total (mg/dL)** | **IgG Total (mg/dL)** |
|---|---|---|---|---|
| 1 | 53.30 | NA | NA | NA |
| 2 | 28.70 | NA | NA | NA |
| 3 | >100 | NA | NA | NA |
| 4 | 50.80 | NA | NA | NA |
| 5 | 20.90 | NA | NA | NA |
| 6 | NA[a] | 15.5 | 447 | 991 |

[a] Values not available

### 3.3.7. Enzyme Linked Immunosorbent Assay (ELISA) to Assess Allergenicity of PGWPH

An indirect ELISA was used to assess the immunoreactivity of WPI, WPH and PGWPH using sera from milk protein sensitive donors as outlined by Brandon et al. (2002), with modifications. Costar 96-well microtiter plates were coated with 100 µL of antigen (5 µg protein/mL phosphate buffered saline (PBS), pH 7.4, either WPI, WPH or PGWPH) overnight at 4°C. Plates were washed 3 times with 300 µL of PBST (PBS with 0.05% Tween 20 w/v) in between each step using an automatic plate washer (Stat Fax, Orlando, Florida, USA). Blocking was carried out for 1.5 h at room temperature by adding 200 µL/well of 3% (w/v) bovine serum albumin (BSA) + 0.05% w/v Tween 20. Sera were diluted (1:10 – 1:50) with PBST to be in the range of 3 to 15 kU of IgE/L. Once diluted, serum (100 µL) was added to each well and the plate was incubated for 1 h at room temperature. Secondary antibody, biotinylated anti-human goat IgE, was diluted (1:250) using tris buffered saline (TBS) (50 mM Tris, 0.138 M NaCl, 2.7 mM KCl, pH 8.0, and 0.01% Tween 20, w/v). The secondary antibody (100 µL) was added to each well and incubated for 1 h at room temperature. This step was followed by the addition of diluted (1:500 in TBS and 0.1% Tween 20, w/v), streptavidin alkaline phosphatase (SAP) (100 µL/well) and incubated for 30 min at room temperature. Lastly, substrate to the SAP enzyme, p-nitrophenol phosphate, (100 µL) was added to each well, and the plate was incubated for 30 min at room temperature. Absorbance was then read at 405 nm using a microplate reader (Biotek, Winooski, VT, USA). To control for non-specific binding of the secondary antibodies, a secondary antibody control was run (Table 6). To serve as a blank, all reagents except the antigen were mixed and analyzed. To serve as a negative control, sera from individuals with no medical history of milk protein allergy were obtained and mixed with the reagents in place of the positive milk protein immunoreactive sera. All analysis was conducted in triplicate. The % reduction in allergenicity of WPH and PGWPH was determined using **Equation 8.** A sample calculation can be found in Appendix E.

**Equation 8:**

$$\%\ \text{Reduction in allergenicity} = 100 \times \left(\text{Corrected ABS}_{\text{WPI}} - \frac{\text{Corrected ABS}_{\text{WPH or PGWPH}}}{\text{Corrected ABS}_{\text{WPI}}}\right)$$

Where:

ABS is the absorbance at 405nm

Corrected $ABS_{WPI}$ is equivalent to $(ABS_{WPI\ positive\ sera} - ABS_{2nd\ antibody})$

Corrected $ABS_{WPH}$ is equivalent to $(ABS_{WPH\ positive\ sera} - ABS_{2nd\ antibody})$

Corrected $ABS_{PGWPH}$ is equivalent to $(ABS_{PGWPH\ positive\ sera} - ABS_{2nd\ antibody})$

Table 6. Reaction mixture for ELISA

| **Assay** | **Antigen[a] (µL)** | **BSA[b] (µL)** | **POS Serum[c] (µL)** | **NEG Serum[d] (µL)** | **2nd Antibody[e] (µL)** |
|---|---|---|---|---|---|
| Blank | — | 200 | 100 | — | 100 |
| 2nd Antibody Control | 100 | 200 | — | — | 100 |
| Negative Sera | 100 | 200 | — | 100 | 100 |
| Positive Sera | 100 | 200 | 100 | — | 100 |

[a] WPI, WPH, or PGWPH (5ug/mL)

[b] Bovine Serum Albumin (3% in 0.05% Tween20)

[c] Sera from individuals with whey specific IgE > 3.5kU/L

[d] Sera from individuals with no medical history of milk protein allergy

[e] Biotinylated Anti-Human Goat Secondary Antibody

### 3.3.8. Statistical Analysis

Analysis of variance (ANOVA) was carried out using IBM SPSS Statistics software version 22.0 for Windows (SPSS, Inc., Chicago, IL, USA). Significant differences among the means of different treatments were determined when a factor effect or an interaction was found to be significant ($P \leq 0.05$) using the Tukey-Kramer multiple means comparison test. ANOVA tables for Chapter 3 can be found in Appendix F (Tables 15-17).

## 3.4. Results and Discussion

### 3.4.1. Determination of Lysine Blockage by Furosine Analysis

The Maillard reaction can have negative impacts on the nutritional quality of proteins, particularly a decrease in bioavailability of lysine as its ε-amino group is very reactive in the Maillard reaction (Finot and Mauron, 1972). Thus, it is important to monitor the extent of blocked lysine in a glycated protein in order to ensure nutritional quality. Initial stages of the Maillard reaction and extent of lysine blockage due to Amadori compound formation can be monitored by the determination of furosine (ε-N-2-furoylmethyl-L-lysine) (Figure 21 in Appendix C), which is formed during the acid hydrolysis of Amadori compounds.

Only 1.00 mg of furosine per g of protein was detected in PGWPH (equivalent to 9.9 mg furosine per g lysine; as the WPH used in these experiments had 10.1 g lysine/ 100 g protein). Given that furosine represents approximately 43.4% of the total Amadori compounds formed under the conditions used for hydrolysis (Krause et al., 2003), the calculated % lysine blockage was 1.31% (on a molar basis). This represents a minimal reduction in bioavailability of lysine. Wang and Ismail (2012) observed a slightly lower value (0.6% lysine blockage in partially glycated WPI after 96 h incubation with dextran (9-10 kDa) (4:1 dextran:protein ratio) at 60°C and 0.49 aw. The higher (almost double) lysine blockage observed for PGWPH in this study compared to that observed by Wang

and Ismail (2012) for partially glycated WPI is likely attributed to differences in protein structure. While WPI is globular, WPH is partially unfolded, making the lysine residues more readily accessible to take place in the Maillard reaction, leading to increased Amadori compound formation and, thus, lysine blockage.

However, other researchers have observed much greater increases in blocked lysine upon glycation. Jiménez-Castaño et al. (2005) observed a 30% lysine blockage in β-lactoglobulin after 48 h incubation with dextran (3.4 kDa) (2:1 dextran:protein ratio) at 60°C and 0.44 $a_w$. Rufián-Henares et al. (2006) reported that incubation of commercial whey protein with dextrinomaltose and heated at 120°C for 10 min resulted in 13.4% lysine blockage. Differences in reported results of lysine blockage can be attributed to glycation conditions as well as different dextrose equivalent (DE) values of carbohydrates used. Glycation using carbohydrates with lower DE-value tend to exhibit less lysine blockage than glycation using carbohydrates with large DE-values, such as glucose or lactose. Also, the protein to carbohydrate ratio affects degree of blocked lysine, with higher ratios exhibiting higher lysine blockage, as observed by Jiménez-Castaño et al. (2005). Overall, the controlled glycation conditions used in this study resulted in minimal effect on nutritional quality, with respect to loss of lysine.

### 3.4.2. Digestibility

To further analyze whether partial glycation had an effect on nutritional quality, *in-vitro* digestibility of WPH and PGWPH was compared. The chromatographic peaks that decreased after digestion had retention times between 22 and 25 min and 39 and 41 min (Figure 17). The areas of these peaks pre and post digestion were used to calculate the percent digestibility for each sample. The digestibility of PGWPH (58.7 ± 3.01%) was not significantly ($P \leq 0.05$) less than that of WPH (65.7 ± 1.14%) indicating that partial glycation did not impact digestibility of WPH. Other researchers have reported that glycated milk proteins, such as glycated bovine serum albumin and β-lg, had a lower proclivity to trypsin digestion due to lower reactivity of trypsin against glycated lysine

and arginine residues (Lapolla et al., 2004; Moreno et al., 2008; Luz Sanz et al., 2007; Corzo-Martínez et al., 2010). With minimal lysine blockage and limited overall glycation of our PGWPH, lower proclivity of trypsin hydrolysis was not significant or apparent based on the digestibility results.

In contrast, glycation of milk proteins has also been reported to increase protein digestion (Chevalier et al., 2001b; Hiller and Lorenzen, 2010; Wang, 2013). For example, Wang and Ismail (2012) reported an 8.9% increase in digestibility of WPI upon partial glycation using dextran (9-10 kDa) due to induced partial unfolding of the protein and, as a result, increased the number of accessible enzyme binding sites. Different reported effects on digestibility could be attributed to differences in the extent of glycation per molecule of protein, which will affect the extent of induced conformational changes to the protein structure, degree of initial denaturation, as well as the extent of steric hindrance preventing digestive enzymes from reaching binding sites.

In this study, glycation was carried out under controlled and mild conditions, and resulted in limited and partial glycation. Such limited glycation would only block a small portion of the digestive enzyme binding sites, resulting in very minimal steric hindrance for the digestive enzymes. In addition, since WPH has already undergone partial unfolding due to enzymatic hydrolysis and therefore already has increased number of accessible peptide bonds susceptible to proteolysis, it makes sense that digestibility of WPH would be minimally impacted by limited glycation conditions used in this study.

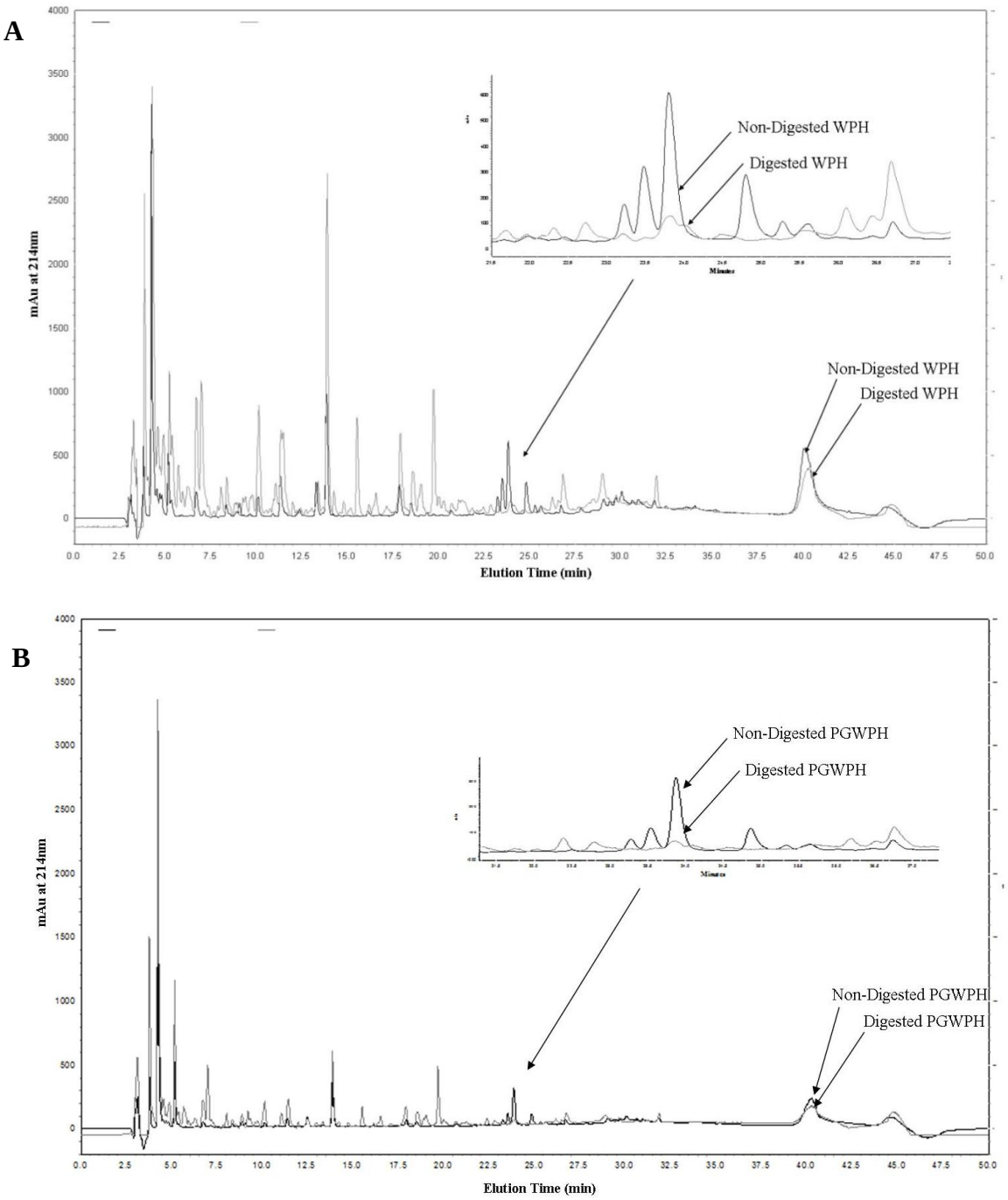


Figure 17. HPLC Chromatograms of WPH (A) and PGWPH (B) before and after *in-vitro* digestion with pepsin and trypsin. Chromatograms show absorbance at 214 nm, scaled to 4000 absorbance units. Additional chromatograms in the upper right corners indicate selected peak areas used for determination of digestibility.

### 3.4.3. Effect of Glycation on ACE Inhibitory Activity

The ACE inhibitory activity of non-glycated WPH and PGWPH pre and post HIC purification was determined in order to assess changes to bioactivity resulting from glycation and potential loss of bioactive peptides during HIC separation. WPH had an average $IC_{50}$ of 0.226 mg protein/mL (Table 7), which fell within the range of $IC_{50}$ values (0.100 and 0.800mg protein/mL) that were reported in literature for whey protein peptides (Pihlanto-Leppala, 2001; Saito, 2008; Malcata, 2013). The $IC_{50}$ value of PGWPH pre and post HIC purification were not significantly ($P \leq 0.05$) different than that of WPH. This study confirms, for the first time, that controlled and limited glycation of bioactive WPH can be carried out while maintaining bioactivity of WPH, as ACE inhibitory activity was maintained upon glycation. Furthermore, the ultrafiltration and HIC separation procedures used to remove free, unreacted dextran from PGWPH was successful in retaining valuable bioactive peptides, as indicated by the lack of significant difference between the $IC_{50}$ values of separated and non-separated PGWPH (Table 7). If glycation of WPH had been more extensive, it is possible that bioactivity could have been negatively impacted. Excessive glycation could interfere with the peptides' ability to bind to the ACE enzyme's substrate binding site and act as a competitive inhibitor. Therefore, limited and controlled glycation conditions are needed to ensure bioactivity of WPH is maintained.

Table 7. $IC_{50}$ value (mg/mL) of WPH and PGWPH

| | WPH ± SE[A] | PGWPH ± SE[A] (pre HIC purification) | PGWPH± SE[A] (post HIC purification) |
|---|---|---|---|
| $IC_{50}$ (mg protein/mL) [B] | 0.226 ± 0.009 [a] | 0.221 ± 0.010 [a] | 0.249 ± 0.005 [a] |

[A] Standard Error

[B] $IC_{50}$: Protein concentration leading to 50% inhibition of ACE activity

[*] Means followed by the same lower case letter are not statistically different according to the Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

### 3.4.4. Effect of Glycation on Immunoreactivity of Whey Protein

Sera from allergenic donors with varying milk protein specific IgE levels were used for quantitative assessment of the immunoreactivity of WPH and PGWPGH. An indirect ELISA was used to obtain quantitative immunoreactivity data as well as assess the overall immunoreactivity of the modified protein. The reduction in immunoreacitivty of WPH and PGWPGH was dependent on the sensitivity of the sera used as different % reductions in immunoreactivity were observed when serum from different participants was used (Figure 18). The observed differences can be attributed to the variation in whey protein specific IgE among the different sera that could react with the epitopes present in the different whey protein samples.

Overall, significant reductions in immunoreactivity were observed for WPH relative to WPI. Upon enzymatic hydrolysis, the immunoreactivity of the protein is altered by cleaving peptide bonds, resulting in the disruption of linear eptiopes, as well as disruption of conformational epitopes due to protein unfolding (L'Hocine and Boye, 2007). Simultaneously, however, some epitopes buried within the interior of the protein may become exposed (L'Hocine and Boye, 2007). Thus, immunoreactivity of WPH will vary among sera used from different participants, as was observed in this study (Figure 18).

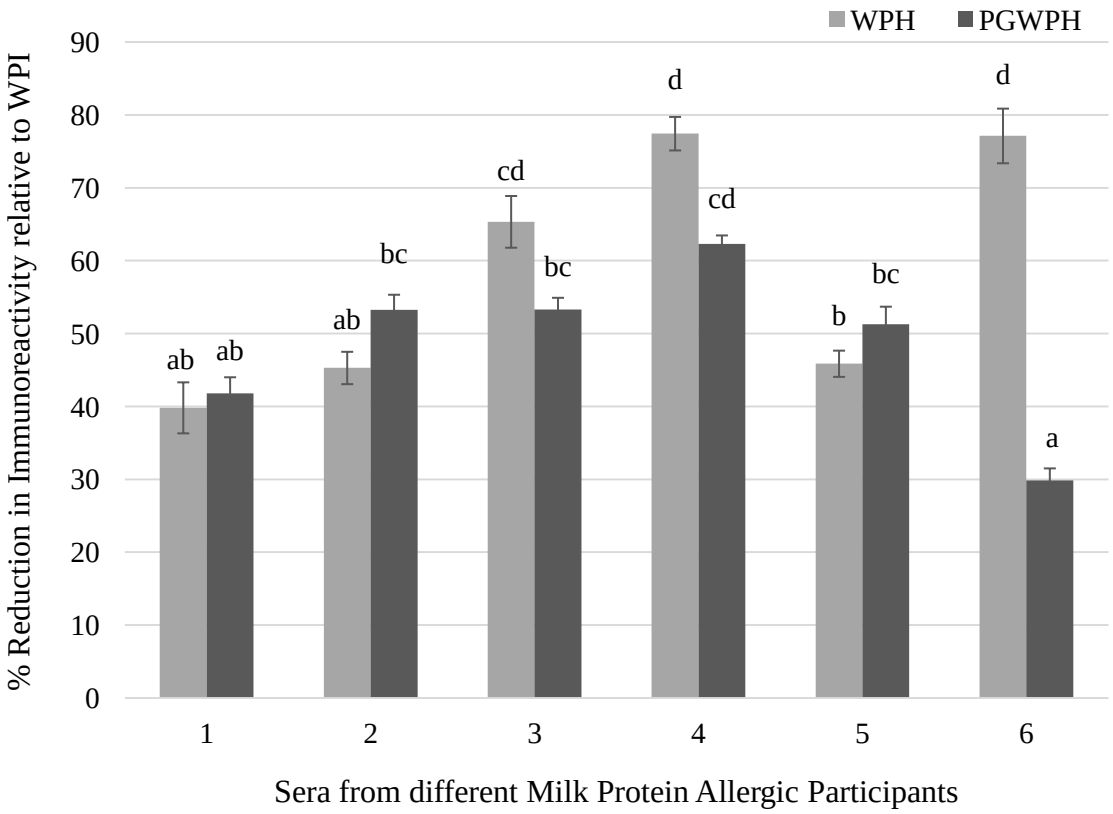


Figure 18. % Reduction in Immunoreactivity of WPH and PGWPH relative to WPI using sera from all participants. Error bars represent standard errors (n=3). Different lower case letters indicate significant differences among WPH and PGWPH according to Tukey-Kramer multiple means comparison test ($P < 0.05$).

Upon glycation of WPH, no significant reductions in immunoreactivity were observed for participants 1, 2, 4 and 5,while significant increases in immunoreactivity were observed for participants 3 and 6 (Figure 18). Contradictory results have also been found by previous researchers. When reduced binding of IgE to glycated allergens was observed, researchers suggested that the reduction was due to carbohydrate masking of the allergen epitopes or induced changes in the tertiary structure of the proteins that disrupt conformational epitopes (Arita et al., 2001; Gruber et al., 2004; Nakamura et al., 2008; Taheri-Kafrani et al., 2009; Bu et al., 2009). In contrast, other researchers who

have observed increased IgE binding to glycated allergens suggested that the Maillard reaction might enhance IgE recognition, or create novel IgE epitopes (Chung and Champagne, 1999; Maleki et al., 2000; Simonato et al., 2001; Pastorello et al., 2002; Nakamura et al., 2005). For example, Maleki et al. (2000) reported that Maillard modified Ara h 1 and Ara h 2 peanut allergens (incubated with glucose, mannose, xylose, or galactose in PBS at 55°C for up to 10 days) had increased IgE binding due to cross-linking to form high molecular weight aggregates, which bound IgE more successfully than unmodified allergens. In addition, Simonato et al. (2001) reported that extracted wheat proteins in baked bread had increased IgE reactivity when it was extensively cross-linked by Maillard adducts. Furthermore, evidence has accumulated that some glycation structures of the Maillard reaction function as immune epitopes. Chung and Champagne (1999) reported that nonallergenic peanut protein (lectin) was converted into a potentially allergenic product upon glycation, as the Maillard reaction products were recognized by IgE from peanut allergic patients when lectin was reacted with glycose or fructose in 0.3 M sodium phosphate at 50°C for 28 days.

It is difficult to give a reason for the contradictory results, as multiple biochemical mechanisms may be at play. However, possible explanations could be attributed to differences in protein structure and size of the carbohydrate used, as those that saw enhancement of IgE binding used smaller sized reducing sugars. Another possible explanation might be due to variation in sensitivity and specificity of whey protein specific IgE in each serum used, as some allergic individuals may have IgE specific for glycated protein structures, while others do not. The results could also be dependent on the extent of glycation and type of carbohydrate used. For example, Bu et al., (2010) reported a 99.8% reduction in allergenicity of β-lg when incubated with glucose at 51.8°C; and 0.79 $a_w$ for 75.7 h. These conditions used were more conducive to the Maillard reaction, likely resulting in more extensive glycation than was observed in our study. On the other hand, Walter (2014) reported an 83 and 82% reduction in allergenicity of soy protein hydrolysate when incubated with dextran at 60°C and 0.49 $a_w$ for 96 h and 120 h, respectively. While these conditions resulted in limited glycation, the

large dextran used could have caused a steric hindrance that resulted in a significant reduction in immunoreactivity. The glycation conditions we used similarly aimed at limiting glycation of WPH, yet no reduction in immunoreactivity was observed. Glycation conditions, therefore, need further optimization. It is apparent that the reduction in allergenicity of a protein via the Maillard reaction can be dependent on multiple factors, namely the structural state of the protein/peptides, type of carbohydrate, reaction conditions (time and temperature) and the resulting extent of glycation. Future work would be directed at optimizing glycation conditions to see if further reduction in allergenicity of WPH could be achieved.

### 3.5. Conclusions

The limited and controlled Maillard conditions utilized in this study preserved the nutritional quality of whey protein hydrolysate upon glycation, as lysine blockage was limited and digestibility was minimally impacted. Additionally, glycation did not affect bioactivity of WPH as ACE inhibitory activity was maintained. Furthermore, the ultrafiltration and HIC separation procedure used to remove free, unreacted dextran from PGWPH was successful in retaining valuable bioactive peptides. Lastly, glycation of WPH did not further reduce allergenicity of whey protein hydrolysate, but future work could be done to optimize glycation conditions for further reduction in allergenicity. Overall, using a combination of enzymatic hydrolysis and limited Maillard-induced glycation may have the potential to produce a functional whey protein ingredient for use in formulating high protein acidified beverages while maintaining nutritional quality and bioactivity.

## 4. Overall Conclusions, Implications, and Recommendations

Controlled and limited Maillard-induced glycation of WPH was successfully carried out under 60°C and 0.49 $a_w$ for 48 h without progression to undesirable advanced stages of the Maillard reaction, as evidenced by the moderate formation of Amadori compounds, low browning and fluorescent compound formation, and only moderate reduction in free amino groups. This work also showed that ultrafiltration and HIC separation can be used to effectively remove free dextran from PGWPH, as evidenced by the high protein yield (88.2%) and the retention of bioactive peptides. Since whey protein and its bioactive properties are currently a popular market trend, bioactive peptides are particularly important to retain during purification of PGWPH. It is also notable that the final protein content of the purified PGWPH (88.4%) is very close to the minimum requirement (90%) for the ingredient to be considered a protein “isolate”. If the extent of glycation were much greater, the protein content of the product would be further decreased, and the product would have to be classified as a “concentrate”. As a result, the ingredient would lose part of its economic value and become less desirable to industry. With the Maillard conditions used in this study, a PGWPH with a 90% protein content could easily be produced that would still be considered an “isolate” and still have benefits of glycation observed in this work. This work showed that partial glycation enhanced solubility and thermal stability of WPH over a broad range of pH, including near the pI, at 5% protein concentration, which is greater than the 4.2% protein concentration required by the FDA to make a "high protein" claim on beverages. Future work could explore solubility and thermal stability at even higher protein concentrations, such as 7% and 10%.

While enhanced solubility and thermal stability are important considerations when selecting an ingredient for use in food applications, nutritional quality is equally important. This work showed that partial glycation minimally impacted lysine bioavailability and digestibility of WPH. Additionally, limited glycation did not impact the bioactivity of WPH, namely its antihypertensive activity. These findings are

important because among the many reasons industry and consumers are drawn to WPH are its rich essential amino acid content, high digestibility and bioactivity. However, partial glycation did not further reduce the allergenicity of WPH. This is attributed to the fact that the extent of glycation is only partial, and that a greater extent of glycation is perhaps needed to see a further reduction in allergenicity of WPH. Further optimization of glycation conditions are hence needed to achieve reduction in allergenicity.

Overall, use of PGWPH as a whey protein ingredient would allow for the formulation of acidified high-protein beverages (> 4.2%). Because PGWPH had enhanced solubility, especially around its pI, beverages could be formulated at pHs around the pI (pH 4.5), which are higher than the pH (3.4) of protein beverages currently on the market, but still considered acidic. As a result, less acid will have to be added to the beverages to reach the target pH and, in turn, beverages sourness and astringency will be reduced, allowing for increased overall consumer acceptance. Consumer demands for an acceptable, nutritional high protein beverage will be fulfilled and consequently the market demand for nutritional whey protein ingredients will increase.

Overall, this study has shown that partial glycation of WPH can be used to create a value-added product for use in high protein beverages. It is recommended that future work be directed at further streamlining the HIC separation process to increasing protein yield and throughput. Also, the enhanced solubility and thermal stability of WPH in this study was obtained using an analytical grade dextran. In order to assess PGWPH in industrial applications, more work would have to be done on creating PGWPH using food grade maltodextrin. Beverage formulation, acceptability evaluations, and storage stability studies of PGWPH produced using maltodextrin would then follow. This collection of future work would help increase industrial feasibility of producing glycated WPH with improved overall quality for use in various applications including, but not limited to, acidified protein beverages.

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## Appendix A. Browning of WPH Incubated with and without Dextran

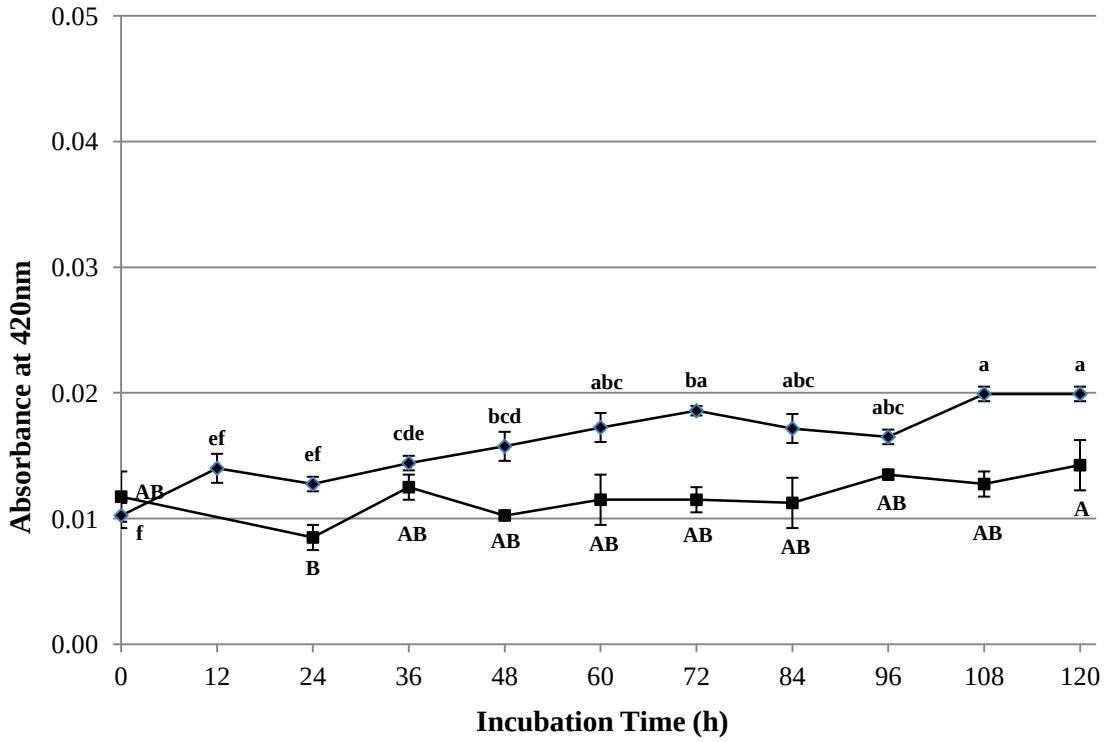



Figure 19. UV-Visible difference spectroscopy absorbance at 420 nm as an indicator of browning of (WPH) incubated with dextran (◆) and control WPH incubated without dextran (■) at 60°C for 0-120 h at 0.49 $a_w$. Error bars for WPH incubated with dextran represent standard errors (n=3). Error bars for WPH incubated without dextran represent standard deviations. Different letters above or below data points indicate significant differences between different time points according to the Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

## Appendix B. Formation of Glycoproteins in WPH Incubated with and without Dextran

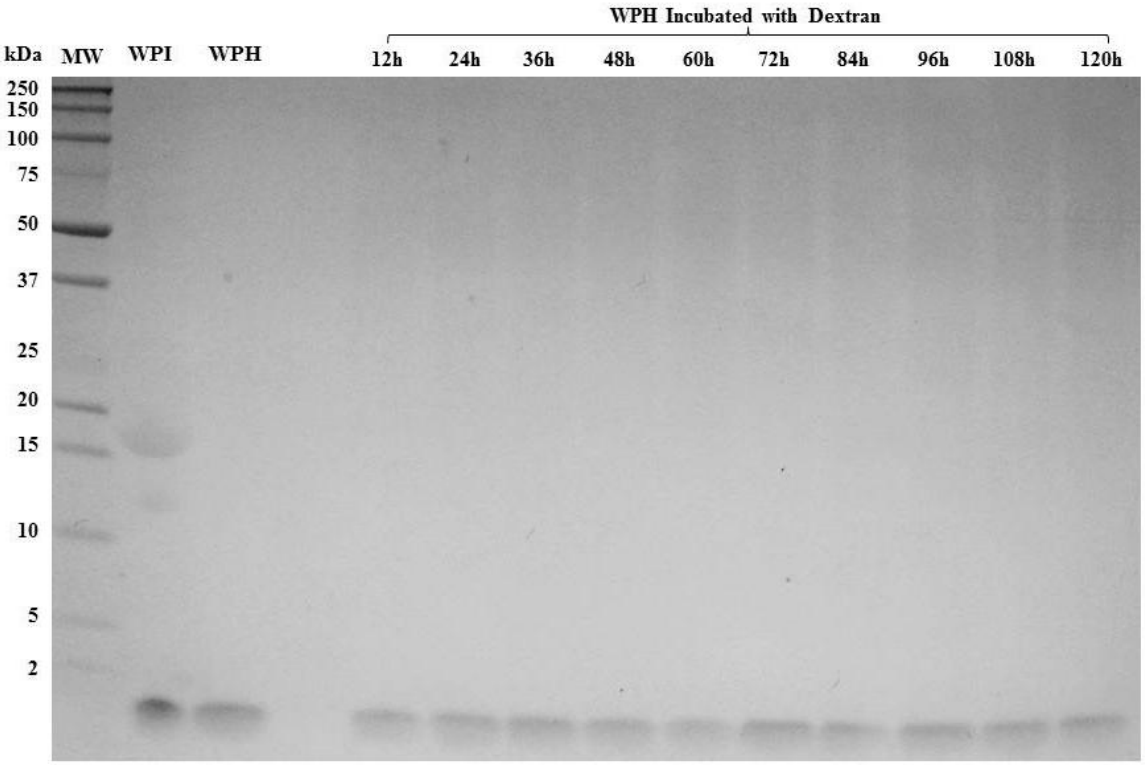



Figure 20. Formation of glycoproteins as visualized by Glycoprotein stained SDS-PAGE for WPH incubated with dextran at 60°C for 0-120 h at 0.49 $a_w$. MW: molecular weight in kDa. Whey protein isolate (WPI) was run as a reference.

## Appendix C. Chromatogram and Sample Calculation for Furosine

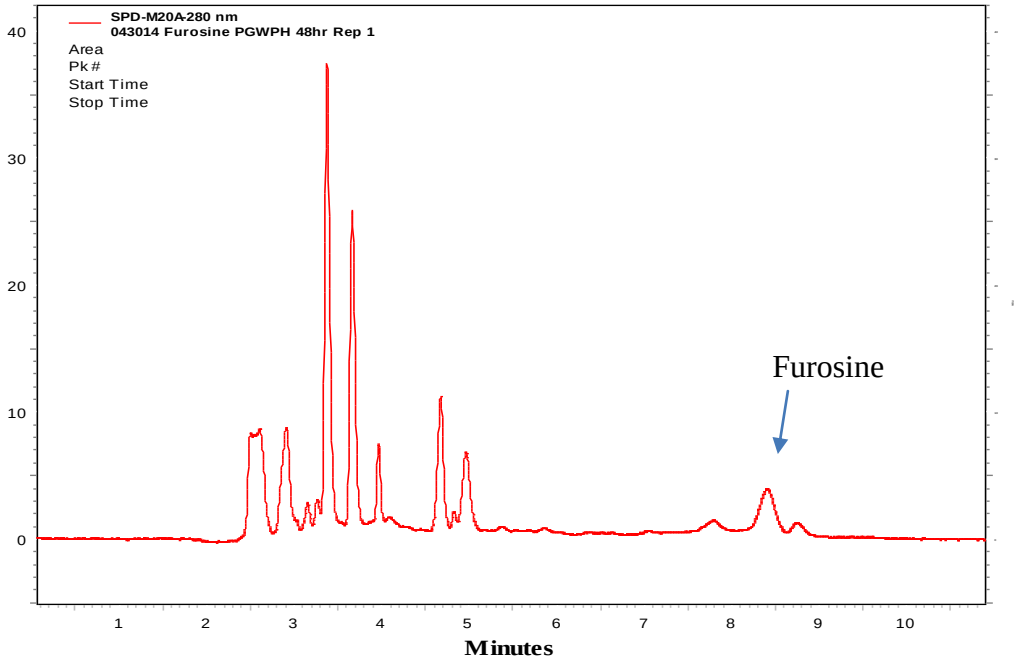



Figure 21. 48 h Incubated PGWPH Sample HPLC Chromatogram. Furosine retention time: 8.320 minutes.

Furosine peak area from PGWPH sample: 54745

Furosine concentration in PGWPH sample using standard curve equation:

$$furosine\ concentration\ (ppm) in\ injected\ sample = x = \frac{(y - 4707.8)}{39340}$$
$$= \frac{(54745 - 4704.8)}{39340} = 1.272\ ppm$$

$$1.272\ ppm = 1.272\ \mu g/mL$$

Thus, in the 2.3mL sample solution from which 20 uL was injected onto the column, furosine content was:

$$\frac{1.272\ \mu g}{mL} \times 2.333\ mL = 2.967\ \mu g$$

This 2.333 mL solution was from 0.5 mL of hydrolyzed solution containing 0.02 g sample protein digested in 3.75 mL of HCl. Thus, furosine content in the original 0.02 g protein is:

$$\frac{2.967\ \mu g\ furosine}{1} \times \frac{1}{0.5\ mL\ hydrolyzed\ solution} \times \frac{3.75\ mL\ HCl}{1} = 22.25\ \mu g\ furosine$$

Equation obtained from furosine standard curve:

$$y = 39340x + 4704.8$$

The molecular weight of furosine is 254. The conversion factor of furosine to Amadori compound to furosine is about 0.434 (Ref). Thus, the amount of amadori compound produced per 1 g of glycated protein is:

$$\frac{22.25\ \mu g\ furosine}{0.02\ g\ sample\ protein} \times \frac{M\ furosine}{254\ g\ furosine} \times \frac{1\ amadori\ compound}{0.434\ furosine\ compound} \times$$

$$\frac{1\ g}{10^6 \mu g} = 10.09\ \mu M\ amadori\ compound\ in\ 1\ g\ glycated\ protein$$

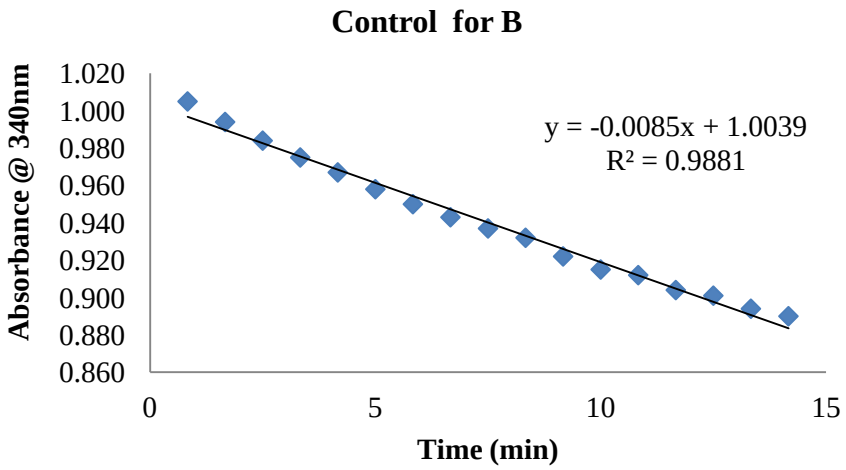
According to the manufacturer, whey protein (the sample protein) is comprised of 10.4% of lysine. Molecular weight of lysine is 146.19. Since formation of amadori compound coinsides with lysine blockage, the lysine blockage in the sample protein can be calculated as:

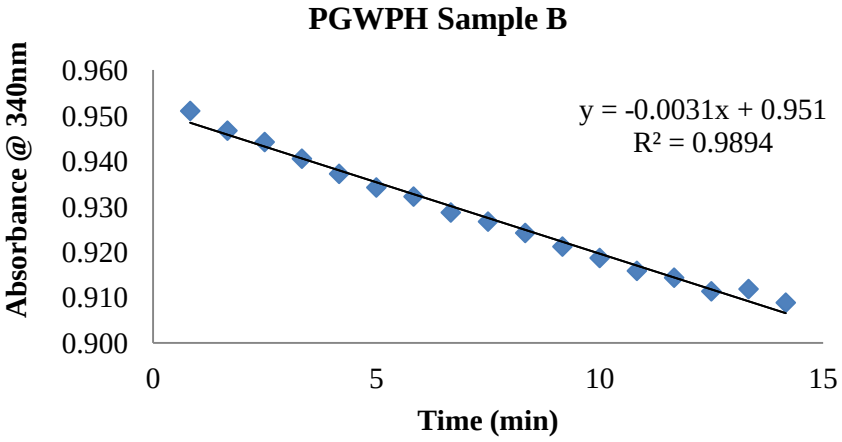
$\%\ Lysine\ Blockage\ (molar\ base)$=

$$\frac{10.09\ \mu M\ amadori\ compound}{1\ g\ sample\ protein} \times \frac{100\ g\ sample\ protein}{10.4\ g\ lysine} \times \frac{146.19\ g\ lysine}{M\ lysine} \times \frac{1\ mM}{1000\ \mu M} \times \frac{1\ M}{1000\ mM} = 1.42\%\ lsyine\ blocked$$

## Appendix D. Sample Calculation for ACE

**Determining % ACE Inhibition:**




$$\% \text{ ACE Inhibition} = \frac{(\text{slope}_{\text{control}} - \text{slope}_{\text{inhibitor}})}{\text{slope}_{\text{control}}} * 100\%$$

$$\% \text{ ACE Inhibition} = \frac{0.082 - 0.044}{0.082} \times 100\% = 45.59\%$$

**Determining protein concentration of the sample:**

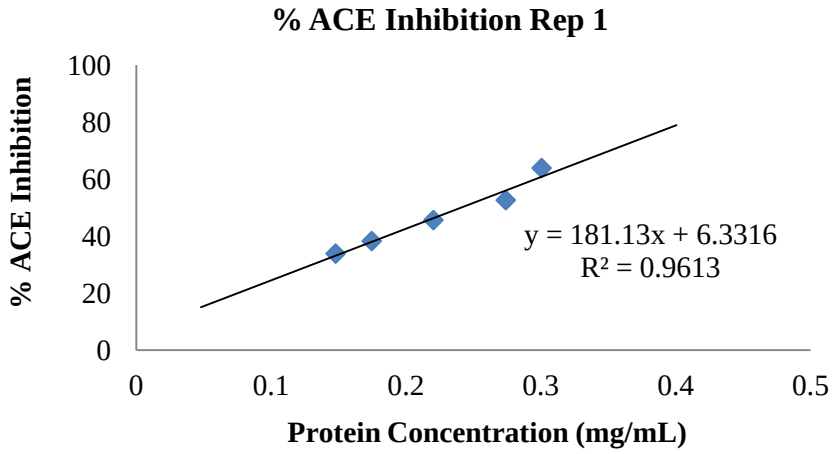
$$\text{Conc. of protein in the well} = \frac{\text{protein concentration from BCA (5.11 mg/mL)} * 10\ \mu\text{L}}{170\ \mu\text{L}}$$

$$= 0.30\ \text{mg/mL}$$

Where:

Protein concentration from BCA (mg/mL) is the protein concentration determined by BCA for each corresponding inhibitor solution added in the ACE assay

**Determining IC50 value:** Once all % ACE inhibitions of each sample have been plotted against their corresponding protein contents, linear regression can be used to obtain equation for the ACE inhibition line from which the $IC_{50}$ value can be determined.




$$y = 181.13x + 6.3316$$

$$IC_{50}\text{value} = x = \frac{(50 - 6.3316)}{181.13} = 0.241$$

Where:

X = protein concentration (mg/mL) of WPH or PGWPH in the well needed for 50% inhibition

y = % ACE inhibition; y = 50 to determine concentration of WPH or PGWPH needed to result in 50% inhibition

## Appendix E. Sample Calculation for % Reduction in Allergenicity

$$\%\ \text{Reduction in allergenicity} = 100 \times \left(\text{Corrected ABS}_{\text{WPI}} - \frac{\text{Corrected ABS}_{\text{WPH or PGWPH}}}{\text{Corrected ABS}_{\text{WPI}}}\right)$$

Where:

ABS is the absorbance at 405nm

Corrected $ABS_{WPI}$ is equivalent to ($ABS_{WPI\ positive\ sera} - ABS_{2nd\ antibody}$) = (0.847-0.073)

Corrected $ABS_{WPH}$ is equivalent to ($ABS_{WPH\ positive\ sera} - ABS_{2nd\ antibody}$) = (0.352-0.075)

Corrected $ABS_{PGWPH}$ is equivalent to ($ABS_{PGWPH\ positive\ sera} - ABS_{2nd\ antibody}$) = (0.291-0.074)

Therefore, % reduction in allergenicity = $100 \times \frac{(0.847-0.073)-(0.352-0.075)}{(0.847-0.073)} = 64.2\ \%$

## Appendix F. Analysis of Variance (ANOVA) Tables for Determining Significant Effects of Treatments

Table 8. Analysis of variance on the effect of incubation time on 304 nm absorbance of WPH incubated with or without dextran.

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| WPH incubated with dextran at 0.49$a_w$, 60°C | Incubation Time | 10 | 0.016 | 316.496 | 0.000 |
| | Error | 51 | 0.000 | | |
| WPH incubated without dextran at 0.49$a_w$, 60°C | Incubation Time | 9 | 0.001 | 27.196 | 0.000 |
| | Error | 10 | 0.000 | | |

Table 9. Analysis of variance on the effect of incubation time on 420 nm absorbance of WPH incubated with or without dextran.

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| WPH incubated with dextran at 0.49$a_w$, 60°C | Incubation Time | 10 | 0.000 | 21.413 | 0.000 |
| | Error | 51 | 0.000 | | |
| WPH incubated without dextran at 0.49$a_w$, 60°C | Incubation Time | 9 | 0.001 | 2.724 | 0.067 |
| | Error | 10 | 0.000 | | |

Table 10. Analysis of variance on the effect of incubation time on % fluorescent intensity of WPH incubated with or without dextran.

| Sample Analysis | Source of Variation | Degrees of | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| WPH incubated with dextran at 0.49$a_w$, 60°C | Incubation Time | 9 | 51274.032 | 110.480 | 0.000 |
| | Error | 20 | 464.103 | | |
| WPH incubated without dextran at 0.49$a_w$, 45°C | Incubation Time | 9 | 11438.132 | 51.509 | 0.000 |
| | Error | 20 | 222.062 | | |

Table 11. Analysis of variance on the effect of incubation time on % free amino group loss of WPH incubated with dextran.

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| WPH incubated with dextran at 0.49$a_w$, 60°C | Incubation Time | 10 | 244.810 | 6.455 | 0.000 |
| | Error | 53 | 37.923 | | |

Table 12. Analysis of variance on the effect of sample type on Solubility of WPH and PGWPH

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| WPH | pH and Heat Treatment | 5 | 2362.018 | 163.108 | 0.000 |
| | Error | 12 | 14.481 | | |
| PGWPH | pH and Heat Treatment | 5 | 82.027 | 2.41 | 0.098 |
| | Error | 12 | 34.036 | | |

Table 13. Analysis of variance on the effect of heating time on Surface Hydrophobicity Index of WPI, WPH and PGWPH

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| WPI | Heating Time | 6 | 3804827.317 | 55.875 | 0.006 |
| | Error | 14 | 66897.667 | | |
| WPH | Heating Time | 6 | 64956.302 | 5.075 | 0.006 |
| | Error | 14 | 12800.429 | | |
| PGWPH | Heating Time | 6 | 29055.889 | 20.245 | 0.000 |
| | Error | 14 | 1435.190 | | |

Table 14. Analysis of variance on the effect of heating time on Total Sulfhydryl Groups of WPH and PGWPH

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| WPH | Heating Time | 6 | 592.636 | 141.501 | 0.000 |
| | Error | 13 | 4.188 | | |
| PGWPH | Heating Time | 6 | 13.871 | 26.088 | 0.000 |
| | Error | 14 | 0.532 | | |

Table 15. Analysis of variance on the effect of sample type on % digestibility of WPH and PGWPH

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| PGWPH, WPH | Sample Type | 1 | 48.606 | 4.686 | 0.163 |
| | Error | 2 | 10.374 | | |

Table 16. Analysis of variance on the effect of sample type on bioactivity (ACE Inhibition) of WPH and PGWPH

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| PGWPH (pre and post HIC purification), WPH | Sample Type | 2 | 0.001 | 2.176 | 0.195 |
| | Error | 6 | 0.000 | | |

Table 17. Analysis of variance on the effect of sample type on % reduction in Immunoreactivity of WPH and PGWPH in relation to WPI for milk allergic subjects

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| PGWPH, WPH | Sample Type | 11 | 644.605 | 23.046 | 0.00 |
| | Error | 24 | 27.970 | | |